A Dissertation

For the Degree of Doctor of Philosophy

Studies on Embryo Development Following Intra Cytoplasmic Sperm Injection and Morphogenetic Analysis of Primordial Germ Cell Migration in Aves

조류의 동결 해동 정자 미세 주입법을 이용한 배아 발생 및 생식세포의 초기 이동 기작에 대한 연구

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이 논문을 농학박사 학위논문으로 제출함

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SUMMARY

Primordial germ cells (PGCs) migrate across the embryo to the gonads where they differentiate and function. Both of morphogenetic and actively through are used for their movement. We have examined the spatial and temporal action of PGCs. As results, PGCs migrated passively toward the anterior region from the preliminary location. However, PGCs and somatic cells are shown different migration speed when they reached the anterior region. PGCs demonstrated markedly faster migration than somatic cells. The results reveal that chicken PGCs use sequential passive and active migration toward the germinal crescent, and only PGCs migrated to germinal crescent.

Cryopreservation of avian semen for preserving the avian genetic resource has been studied for more than 80 years, and there have been many technical difficulties due to the complexity of avian reproductive system. We found that the mixture shown the highest fertility composes of 8% glycerol with 3% DMA in prefreezing diluents. However the mixture was a harmful effect with the addition of cryoprotectant especially with glycerol. Our results show that the sperm preserved in the mixture composed of 6% glycerol containing 5% DMA had 5% less motility. Thus we used that mixture and the sperm preserved in the mixture produced fertilized eggs. Additionally, we tested incubation time for glycerol removal by monitoring under scanning electron microscopy (SEM). We found that complete removal of glycerol requires at least 30 min incubation time.

We have investigated the ability of cryopreserved/thawed quail sperm by intracytoplasmic injection to the unfertilized ovum. An injected egg was incubated with egg shell surrogate system. We have used both PLC zeta (PLCζ) and inositol 1,4,5-trisphosphate (IP_3). Embryo development ratio

increased significantly compared to fresh sperm only (90% vs. 13%).

Avian species surrogate egg shell system has been adapted in many different experimental fields. However, the viability needs to be improved and the system should be more simplified for commercial uses. We have established the quail egg surrogate system for embryo developmental study. The system has produced high percentage of hatchability in both thick albumin capsulated and non-capsulated egg, 78% and 60% respectively. Furthermore, we have succeeded in higher hatchability in single cell stage of embryo incubation.

This study could provide deeper understanding of germ cell movement mechanism in early embryo developmental stage. And, we tested for ICSI by producing chick from cryopreserved sperm for avian genetic resource conservation. We have established knowledge in avian embryo development to understand embryo fertilization and developmental stratagem for atmosphere of ex ovo culture system used by surrogate egg shell system complementation. This study could bring deeper understanding in avian culture system with germ cell migration and possibilities of hatching chick by ICSI and surrogate egg shell system for conservation of genetic resource in the future.

Keywords: Primordial germ cells migration, cryopreservation of avian semen, intracytoplasmic sperm injection, surrogate egg shell system

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BSA	bovine serum albumin
AI	artificial insemination
cDAZL	chicken deleted in azoospermia-like
cDNA	complementary DNA
CMV	Cytomegalovirus
cRNA	complementary RNA
DAPI	4′,6′-diamidino-2-phenylindole
DF-1	a continuous chicken embryonic fibroblast cell line
DIG	Digoxigenin
DMA	dimethylacetamide
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EG	ethylene glycol
EGK	Eyal-Giladi and Kochav
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HH	Hamburger and Hamilton
ICSI	intracytoplasmic sperm injection

IP3	D-myo-inositol 1,4,5-tris-phosphate trisodium salt
KO	Korean Oge
MA	N-methylacetamide
MACS	magnetic-activated cell sorting
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PGCs	Primordial germ cells
PKH	Paul Karl Horan, PKH dyes
PMZ	posterior marginal zone
PVM	perivitelline membrane
RT-PCR	reverse transcription-polymerase chain reaction
SAS	Statistical Analysis System software
SEM	Scanning electron microscopy
SGP-1	Prosaposin
SST	sperm storage tubules
TG	Transgenic
TNF	Tumor necrosis factors
UVJ	Uterovaginal junction
WL	White Leghorn
ZP1	Zona pellucida sperm-binding protein 1
ZP3	Zona pellucida sperm-binding protein 3
ZPC	Zona pellucida glycoprotein C

CHAPTER 1

GENERAL INTRODUCTION

The developmental environment in avian species embryo is different from the mammalian one. Embryo of avian species develop outside of the mother's body, and whole nutrition is supported by yolk with germ free circumstance**.** The main purpose of this study is to understand the avian fertilization, primordial germ cell (PGC) migration and embryo development by using avian egg shell surrogate system on ex ovo culture, intracytoplasmic sperm injection (ICSI) and PGC migration study. To understand embryo development in avian species, we used several different techniques, for example surrogate egg shell incubation for observation of embryo development and embryo survival environment study and ICSI to understand of fertilization and next PGC migration for germ cell migration activity in early embryo development.

Avian species embryo culture system by surrogate egg shell incubation have been applied to various studies in avian species, for example avian species embryo development study, transgenic avian production and avian tissue development study (Byun et al., 2011; LaRue et al., 2003; Ono and Wakasugi, 1983). The surrogate egg shell culture system was completed in 1988 by Perry (Perry, 1988a). An avian embryo culture system for single cell stage to hatch was used to understand avian species embryo development and understanding of cell pluripotency, PGC migration study ,embryo formation, xenotransplantation, tissue interaction, transgene, and many other experimental fields (Brand-Saberi et al., 1996; Diez-Fraile et al., 2010; Guinazu et al., 2007; Mozdziak et al., 2006; Ono et al., 1994; Perry, 1988b; Petitte et al., 2004).

The Nagoya protocol on access to genetic resources and the fair and equitable sharing of benefits arising from their utilization was made in connection with the Conference of the Parties to the Convention on Biological Diversity at its 10^{th} meeting on October 2010 in Nagoya, Japan. The Nagoya protocol is an international agreement which aims for sharing appropriate access to genetic resources, appropriate transfer of relevant technologies, and appropriate funding thereby contributing to the conservation of biodiversity **(**Protocol: Booklets

http://www.cbd.int/abs/doc/protocol/nagoya-protocol-en.pdf).

Importance of avian species has become more considerable in animal genetic resource conservation. Last several decades a lots of avian genetic stocks has disappeared (Fulton and Delany, 2003). Especially last few years, avian influenza has been spread all around world, consequently researchers has worked on such sufficient protection methodology that has been obligated, and also animal genetic resource preservation has been regarded important in the future of animal safety. Avian genetic resource is preserved in the form of livestock, sperm cryopreservation and PGC cryopreservation (Blanco et al., 2000; Kino et al., 1997).

Avian genetic resource conservation with sperm cryopreservation method has been studied for several decades for genetic resource conservation. Shaffner has succeeded in production of fertilized eggs from artificial inseminations with cryopreserved avian semen without hatched chick in 1941 (Shaffner, 1941). Polge has found that glycerol can be used as a cryoprotectant for cryopreservation to produce of fertilized egg (Polge et al., 1949). During the 1980's cryopreservation method has been developed rapidly (Bellagamba et al., 1993; Blesbois et al., 2004; Donoghue and Wishart, 2000; Lake, 1986; Surai and Wishart, 1996; Tselutin et al., 1995). Several different types of cryoprotectants such as Dimethylacetamide (DMA), Dimethyl sulfoxide (DMSO), Dimethylformamide (DMF) and Ethylene glycol (EG) been used for avian species sperm cryopreservation. Recently, cryopreservation of avian semen became more considerable in genetic resource preservation such as endangered avian species and transgenic fowls.

ICSI method has been established for several animals including avian species. It was regardless of their systemic difficulties in fertilization system. Recently avian species ICSI system has been established with high rate of fertilization with genetic manipulations (Hrabia et al., 2003; Mizushima et al., 2007). It would be considerable method for avian genetic resource conservation. ICSI is an important technique in animal biotechnology for animal cloning and

conservation of genetic resources. It has been challenging in avian species. In this study, we investigated the ability of cryopreserved/thawed quail sperm to activate fertilization and embryo development. Cryopreserved/thawed sperm combined with ICSI and following surrogate eggshell culture could be significantly important for development and conservation of avian study field.

Avian PGCs are originated from the epiblast (Swift, 1914). PGC differentiation occurs by preformation and induction. The PGC actively migrates and colonizes in the gonad (Ginsburg and Eyal-Giladi, 1986). The PGCs are differentiated, and result in the sexual differentiation of spermatogenesis or oogenesis (Swift, 1914). PGCs have unique characteristics while it is in development and generate functional gametes.

PGC is the precursor of functional gametes in most animals. In initial developmental phase, PGC segregate from somatic cell lineages. Most of mammalian species PGCs originate from an extragonadal region. And they migrate across the embryo to the gonads. However, avian species need understanding of PGC migration for the spatial and temporal action. Thus we tested PGC migration toward the anterior region of the embryo with exogenous PGC spatial and temporal action. In this study we found that avian PGCs undertake passive migration toward the anterior region. PGCs start active migration after they reach the germinal crescent.

In this study, there are three different sessions for individual different experiment. Avian species surrogate egg shell incubation system is addressed in chapter 3 to understand avian species development from single stage to hatching. Chapter 4 is avian species ICSI system with cryopreserved/thawed sperm to produce of fertilized ovum. To understand PGC migration, experiment placed in chapter 5 in early avian species development with germ cell migration. The conclusion of this study is chapter 6 and Summery of Korean is chapter 7.

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CHAPTER 2

Literature Review

1. Experimental animal

1.1 Korean Oge (KO)

Korean Oge (KO) chickens were previously characterized to investigate its hereditary characters and some economical traits (Han *et al.* 1988b). However it has not been registered as new breed because of fewer studies on characteristic feature.

The genetic background of KO was biased on breeding. General description of KO chickens are: short rectangular shaped body with six to eight pointed stands up straight reddish black color comb on the head; black colored with a white tip medium length of beak; round and black eyes; reddish black and featherless face; small and varied colors of ear lobes; medium size and elongated reddish black wattles; greyish black tongue; shank of medium length with four greyish black toes; the body covered by dark black feathers; greyish black skin; and greyish black bone cortex (Nahm 1997).

The genetic characteristics of KO chickens are a highly inbred line and closely related to the Korean native yellow fowl by DNA fingerprinting study (Lee *et al.* 1995). The native KO chickens share the features of Japanese, Chinese and Indian chicken populations which represent the high genetic variability (Lee *et al.* 2007).

1.2 White Leghorn (WL)

White Leghorn (WL) chickens are the most commonly known avian breed for egg laying and meat production.

WL chickens were first reported in Tuscany in Italy and exported to North

America. WL chickens have single comb, legs are yellow in color, and ear-lobes are white in color. It was tested for environmental stress effect for their body weight growth (Gross & Siegel 1980).

WL chickens are also very good resource for genetic studies such as quantitative trait locus (QTL) (Tsudzuki *et al.* 2007a, b; Ankra-Badu *et al.* 2010; Podisi *et al.* 2011), genetic differentiation (Tadano *et al.* 2011a, b), and gene polymorphism (Cao *et al.* 2007).

1.3 Japanese quail

The Japanese quail is Phasianidae family and scientific name is Coturnix coturnix japonica. Japanese quail eggs are laying small about 30 mm in length and weighing average 10 g. The body weight of Japanese quail is around 127g and incubation to hatch takes approximately 16.5 days.

The history of Japanese quail has introduced around 11th or 12th century China to Japan. Nowadays it has used for egg production which was originally introduced for the purposed of song bird.

The first time for research model used Japanese quail by Padgett & Ivey in 1960 (Padgett & Ivey 1960). The Japanese quail has been used for comparative study studies in chick, turkey, duck, goose and guinea fowl embryo developmental stages (Sellier *et al.* 2006).

2 Avian embryo surrogate egg shell incubation system

2.1. An avian surrogate egg shell incubation system

An avian surrogate egg shell incubation system is using recipient egg shell for incubate early developing embryo. The developmental stratagem of early embryo between mammal and avian species are different in the developmental atmosphere. An avian species embryo growth in the egg shell and nutrition are supported by yolk deposited inside of it. An egg shell protects the developing embryo and air space supporting for shock absorbance and embryo air supporting. This optimized shelter provides perfection of environmental for growth of embryo**.**

2.2. The history of avian embryo culture system

There was number of different avian embryo culture system established by numerous of research groups. It is depends on culture system, tool, environment and days of incubation start. Initially, egg shell culture system used from 1960 to 1994 (Hamburger 1960; Perry 1988a; Naito & Perry 1989; Ono *et al.* 1994). The shell-less culture system has used for several purpose of studies for in vitro culture system confirmation, long term culture system with hatching (Romanoff 1943; Corner & Richter 1973; Auerbach *et al.* 1974; Ono & Wakasugi 1983b).

The first complete method of surrogate eggshell culture with single cell stage of egg was developed by Perry in 1988 (Perry 1988a). This complete culture system used single-cell stage ovum to hatch. Three different sequential systems were used for avian embryo culture (Perry 1988a). Firstly, the system I for fertilized ovum culture to allowed blastoderm formation within 24 hr in CO2 incubator. And next, the function of system II used for 3 days in complication of embryogenesis in surrogate egg shell. Finally, incubated embryo was transferred to bigger chicken

eggshells for growing and hatching. This technique was applied to embryo development, transgenic animal production and tissue development in avian species (Ono & Wakasugi 1983a; LaRue *et al.* 2003; Byun *et al.* 2011).

The cultures of embryo in the shell were established by many groups (Hamburger 1960; Ono & Wakasugi 1983b; Perry 1988a; Naito *et al.* 1990; Ono *et al.* 1994). However, the cultures of single cell stage embryos were produced within low hatchability (Perry 1988b; Tomao Ono 1996). The first time of long term culture for avian embryo was reported by Barnett, however, hatched chick was not produced (Barnett 1982).

2.3. The surrogate egg shell incubation extension and improvement

The first completion of avian surrogate egg shell culture system has low as 7% of hatching rate (Perry 1988a). This manipulation technic has improved 34% in 1989 and 1990 (Naito & Perry 1989; Naito *et al.* 1990). Naito has completed avian embryo culture system with egg taken from magnum (Naito *et al.* 1990). This system has adapted in quail surrogate egg shell incubation system (Ono *et al.* 1994; Naito 1995; Kamihira *et al.* 1998). The preincubated embryo for two days and embryo were transferred to surrogate egg shell or artificial shell with calcium lactate or egg shell powder (Borwompinyo *et al.* 2005).

2.4. The fertilization and development of avian embryo

The fertilization of avian ovum take place in the infundibulum after ovulation and start to secreted albumen and the shell upon it (Olsen & Neher 1948). After ovulation, it takes 4.5 hr for first cleavage in shell gland (Hamburger & Hamilton 1992). Around 50,000 to 60,000 cells composed in the embryo (Spratt & Haas 1961).

An avian egg is fertilized in the infundibulum after ovulation. After 3 hours spent in the oviduct tract, it is start to development with pronuclei fuse (Perry 1987). An oocyte moves among the oviduct tract for 23-24 hours before oviposition in infundibulum, magnum, isthmus and shell gland. In the magnum, egg yolk is surrounded by albumens within 3 hours. And shell membrane is secreted in isthmus for 1.5 hour. An ovum spends approximately 18-19 hours in the shell gland for egg shell formation.

The process of chicken embryo development for day 1 shows embryonic tissue. The 2^{nd} day of egg incubation demonstrates for blood vessels. The embryo heat beat is start at 3^{rd} days of incubation and 4^{th} day of incubation is timing for growth of eye parts. 5^{th} and 6^{th} day of embryo incubation is starting to see elbows, knees and beak. The feather tracts are visible in 8^{th} day and the first few feathers are visible in 12^{th} day. The embryo gut is drawn to abdominal cavity with in 15^{th} day. The embryo is almost completely growth in 18 days of incubation. The day of 20 days of incubation is time of pipping in internal and external. Finally, chick is hatched out 21^{st} day (Hamburger & Hamilton 1992).

2.5. The uses of avian embryo culture system

The avian embryo ex ovo culture system has used various field of study. In 1988, Perry have used surrogate egg shell incubation system for embryo developmental study (1988). Embryo ex ovo culture system has used various fields in avian species embryo development, transgenic animal production and tissue development study (Ono & Wakasugi 1983a; LaRue *et al.* 2003; Byun *et al.* 2011). This technique has applied to producing transgenic animal, embryo formation, xenotransplantation and tissue interaction (Perry 1988b; Ono *et al.* 1994; Brand-Saberi *et al.* 1996; Guinazu *et al.* 2007; Diez-Fraile *et al.* 2010).

The shell-less culture system were used for several experiment (Romanoff 1943; Auerbach *et al.* 1974; Ono & Wakasugi 1983b). The embryo culture with vitelline membrane methods has used for several purpose of experiment (New 1955; Flamme 1987). A plasma or agar clot were used for avian embryo culture system as well (Spratt 1947b, a; Eyal-Giladi *et al.* 1994; Schoenwolf & Yuan 1995).

3. An intracytoplasmic sperm injection (ICSI) method for avian species

3.1. The history of ICSI method and application

It has established for several animals for intracytoplasmic sperm injection (ICSI) method. The first mammalian egg injection was established by Lin (Lin 1966). In 1998, first live rabbit was produced by Yoshimura et al (Yoshimura *et al.* 1988).

ICSI technique was used for preservation of important genetic resource in domestic and wild livestock species (Keskintepe *et al.* 1997). The DNA carrier used by sperm was generated transgenic livestock on ICSI technique (Perry *et al.* 1999; Perry *et al.* 2001). The pharmaceutical proteins could be produced by ICSI (Keefer 2004).

Timson and McDermott (1994) were used micromanipulation tools in marine animals for ICSI (Timson & Mcdermott 1994). The first succession of ICSI was reported by Hiramoto with sea urchin in 1962 (Hiramoto 1962). This technique was established in amphibians by Graham (Graham 1966). The first live rabbit was produced with ICSI-induced fertilization by Yoshimura (Yoshimura *et al.* 1988).

3.2. An ICSI method for avian species

Recently, avian ICSI system has established with high rate of fertilization in other gene manipulations (Hrabia *et al.* 2003; Mizushima *et al.* 2007). An artificial insemination using cryopreserved sperm is not practical due to variable fertility rate in bird. It has succeeded early embryonic development with ICSI in quail even there were systemic difficulties in fertility system (Hrabia *et al.* 2003).

Unfertilized eggs from a female quail soon after oviposition and next ovulation are obtained. The accurate time of ovulation is obtained by a regular laying

which is monitored by a videotape recording, sperm are collected from male, diluted, and transferred in the medium filled plate. Our group was used as intact sperm and the other is frozen and kept in the liquid nitrogen for a week. Under the inverted microscope s single sperm is isolated with a fine glass capillary pipette and injected in to central area of blastodisc of oocyte under the stereomicroscopy. Frozen sperm is thawed at room temperature, and injected into oocyte. Injected oocytes are incubated under 10 % CO2 for 24 hours to further fertilization (Hrabia *et al.* 2003; Mizushima *et al.* 2007; Mizushima *et al.* 2014).

The stages of blastodermal and embryonic development were classified according to Eyal-Giladi and Kochav under a stereomicroscope (Eyal-Giladi & Kochav 1976). To screen for true development of embryos with many nuclei, blastoderms were stained with 4,6-diamidino-2-phenylindole (DAPI), a DNA-specific dye, as a whole mount as described by Waddington (Waddington *et al.* 1998).

3.3. An ICSI method with cryopreserved sperm in avian species

There was a not significantly different result between fresh and cryopreserved epididymal versus testicular spermatozoa for ICSI (Desai *et al.* 2009). The fertilization and embryo developmental rates are not affected by oocyte vitrification procedure followed by ICSI (Rienzi *et al.* 2011). Intracellular calcium might relate to oocyte activation and blastodermal development (Mizushima *et al.* 2007).

Evaluation of the fertility of cryopreserved-thawed sperm in quail oocyte: It is critical that the cryopreserved-sperm lacks consistent mobility causing reduction of fertility rate after thawing. If thawed sperm is fertilizable with ICSI, this sperm can be used for protection of endangered species and production of transgenic bird in future. In addition, the oocyte activation mechanism in birds is fascinating subject

that has never been explored.

3.3.1 Avian sperm cryopreservation

Large numbers of avian species are in decline and endanger in the world caused by natural selection, inbreeding and disease. Especially, an avian influenza has been spread all around world.

There are few animal genetic resource conservation methods been analyzed, such as the maintenance and periodic reproduction of live animals, semen cryopreservation, cryopreservation of the cells from an avian blastodisc, cryopreservation of primordial germ cells (PGCs) more specifically, preserved tissues from which DNA can be isolated, as cloned genomic or cDNA, or as nucleotide sequence data can be using conservation and future genetic recovery (Brillard & Bakst 1990; Buss 1993; Naito *et al.* 1994a; Donoghue *et al.* 1995; Kino *et al.* 1997). The important to sustain the conservation of genetic resource of avian species is essential manner for biodiversity.

Biodiversity is a common theme in the conservation of all types of avian genetic resources but the strategies used to meet this goal could be difficult. The goal of avian genetic resource conservation is the maintenance of genetic integrity of a species or populations within an avian species. However, the avian sperm cryopreservation has studied with many groups in difference of freezing and thawing method, difference of cryoprotectant which were carried out differences of fertility and hatchability. As a result, there were not sufficient results for commercial uses of fertility until now.

Recently, researchers were spent several years to overcome particularly jeopardy avian influenza for avian species existence. However, there are not vibrant

ways to surmount avian influenza which are extremely danger both avian and human.

Cryopreservation for live cell has been studied nearly century. Nonetheless many research groups are looking for competent methodology for cryopreservation technology in animal cell cryopreservation such as more effective cryoprotectants and freezing / thawing methods (Lake 1986; Alexander *et al.* 1993; Bellagamba *et al.* 1993; Surai PF 1996; Donoghue & Wishart 2000; Blesbois *et al.* 2007).

3.3.2. The purpose of avian sperm cryopreservation

More than 80 years, it has studied for avian species cryopreservation. However, numbers of researchers are projected to efficient methodology and convinced cryoprotectant. Around 500 avian species are endangered or critically endangered in the known 9,672 existing avian species of about 5% (Blanco *et al.* 2009). Recently, Importance of avian semen cryopreservation became more considerable not only genetic resource preservation but also livestock maintenance of expenses matter. Especially, avian influenza has spread all around world in threat to avian species.

The maintenance of livestock in avian species is time limited and consuming enormous amount of money and space. However, cryopreserved semen is more effective at time and place limitation. And frozen semen is possibly transported in the domestic region and international country to country easer then livestock (Hammerstedt *et al.* 1990). The sperm cryopreservation is important in genetic diversity conservation (Critser J. K. & 2000). It was published in chicken sperm cryopreservation succession by Lake and Ravie (Lake & Ravie 1979).

3.3.4 The history of sperm cryopreservation for artificial insemination (AI)

In the 1950s, AI method has started to use the bull sperm. The primary method of AI was experienced by bitch and produced of 3 pubs in 17 (Herman 1981). Spallanzani and John Hunter was established that first of AI in human pregnancy (Herman 1981). The first of AI calf was born at the Oklahoma Experimental Station in 1907 (Herman 1981).

The cryobiology and cryopreservation have firstly established at the 1600s (Sherman 1964). It was used for animal production. The standard cryopreserved sperm AI methods has used by egg yolk–glycerol for freezing bull sperm (Foote 1970). In the bore sperm, it has used with pelleting method for AI (Pursel & Johnson 1975). The mouse sperm was studied and successfully cryopreservation and produced by three different groups (Okuyama M *et al.* 1990; Tada *et al.* 1990; Yokoyama M *et al.* 1990).

The avian species sperm cryopreservation is initial technology of avian genetic source conservation for importance of avian genetic resource protection. In 1941, Shaffner produced fertilized eggs from artificial inseminations with cryopreserved avian semen (Shaffner *et al.* 1941). However, there was no hatched chick been produced. The glycerol can be used cryoprotectant for cryopreservation in 1949 by Polge (Polge *et al.* 1949). It has been very rapidly developing for cryopreservation and thawing method (Lake 1986; Bellagamba *et al.* 1993; Surai & Wishart 1996; Donoghue & Wishart 2000).

3.3.4. The uses of cryoprotectant for avian sperm

Polge et al. (1949) established that glycerol could be used for cryoprotectant (Polge *et al.* 1949). Cryoprotectants have developed in

cryopreservation methods with several types glycerol, dimethylacetamide (DMA), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and ethylene glycol (EG) and different type of sperm packaging (Tselutin *et al.* 1995; Tselutin *et al.* 1999; Woelders *et al.* 2006).

Glycerol has used for avian semen cryoprotectant by Lake and Ravie in 1979 (Lake & Ravie 1979). Sexton used dimethyl sulfoxide (DMSO) as internal cryoprotectant for cryopreservation (Sexton 1980). Dimethyl formamide (DMF) and dimethyl acetamide (DMA) were used for semen cryopreservation (Schramm 1991; Tselutin *et al.* 1995).

The first succession of chicken semen cryopreservation published by Lake and Ravie (Lake & Ravie 1979). It has succeeded in blue rock pigeon (Columba livia) artificial insemination with cryopresered sperm (45%) (Sadanand D. S. 2004).

It was established that the best cryoprotectant for semen cryopreservation is glycerol. However, the glycerol for uses of cryoprotectant is harmful effect to sperm (Tselutin *et al.* 1999). And the left of glycerol on sperm serface is contraceptive effect of fertilization for intercept of the sperm storage tubules (SST) settlement even only 1% of sperm reached at SST in natural fertilization (Marquez & Ogasawara 1977).

3.3.5. Avian semen cryopreservation methods

There are main difficulties in semen cryopreservation on cooling/freezing and the thawing procedure (Tselutin *et al.* 1995; Tselutin *et al.* 1999; Woelders *et al.* 2006). First of avian semen cryopreservation method was used low cooling, freezing and thawing in glass vials with glycerol by Lake and Stewart (1978).

Next improved method has established by Sexton in slowly and straws pakage with DMSO (Sexton 1980). However, there were pellets packaging methods with DMF (Schramm 1991) and DMA (Tselutin *et al.* 1995).

3.3.6. The mechanism of fertility capacity in sperm quality

The sperm velocity and linearity contribute to overall sperm mobility phenotype and important characteristics of turkey sperm function (King *et al.* 2000). There was not strong relation between sperm competition with both sperm swimming speed and sperm length (Kleven *et al.* 2009).

The storage of sperm detects damage and sensitive in motility or fertility (Kasai *et al.* 2000). Heat stress gives decease of male chicken fertility by decreasing seminal plasma and intracellular ion concentrations (Karaca *et al.* 2002). Uterovaginal junction (UVJ) of the turkey accepts sperm more readily or store sperm more efficiently than SST (Bakst & Vinyard 2002).

Anti-sperm immunoresponses in hen oviduct relate to the sperm survivability (Łukaszewicz E. 2008). The increase of interleukin-1b and lipopolysaccharide-induced TNF factor relate to the sperm degradation and elimination in utero-vaginal junction to involve in sperm to survive in sperm storage tubules (Das *et al.* 2009).

The osmotolerance is very important in sperm survival under hypertonic conditions with the way of temperature and time of exposure (Blanco *et al.* 2008). The capacity of resist oxidative stress might strong in colorful male sperm, carotenoid antioxdents (Helfenstein *et al.* 2010).

The sperm size traits were negatively associated with the duration of sperm

storage but it was independent of the risk of sperm competition estimated from relative testis mass (Birkhead & Immler 2007; Immler & Birkhead 2007).

3.3.7. The mechanism of avian species oocyte fertilization

Spermatozoa in binding to the inner vitelline membrane support by sperm-associated bodies which making holes in the vitelline membrane and passing through sperm in to oocyte (Rabbani *et al.* 2006). The stimulation of motility and acrosomal exocytosis could be interacting with inner perivitelline layers $Ca2^{+}$ (Ashizawa *et al.* 2006).

The regulation of the acrosome reaction required for Protein phosphatase-type 2B (Ashizawa *et al.* 2004). N-linked glycans on ZP1 activate acrosome reaction in Japanese quail (Hanafy *et al.* 2007; Sasanami *et al.* 2007). The PKA, PIK3, and MAPK1 pathways could be involved in Acrosome Reaction (Lemoine *et al.* 2009).

The embryos fertilization-like activation of bovine SCNT was derived by PLCζ. Thus, the function of PLCζ enhances nuclear reprogramming (Ross *et al.* 2009). Sperm fact and PLCz immunoreactivity enhanced oscillation-inducing factor in mammalian fertilization (Yoon & Fissore 2007). Use of chicken PLCζ triggers egg activation in mouse oocytes (Coward *et al.* 2005). The oviductal fluid has involved in membrane destabilization prior to fertilization by the phospholipase activities (Douard *et al.* 2004).

Sperm-egg binding improves fertility by fragment of prosaposin (SGP-1) (Hammerstedt *et al.* 2001). Avian β-Defensin 3 protects the sperm from the microbial infection in the male and female reproductive tracts (Shimizu *et al.* 2008). The function of N-linked glycans is associated to sperm–egg interaction in chickens (Robertson *et al.* 2000).

The function of ZP1 is the inhibition of sperm binding to the PVM and also ZPC relates with function of sperm and egg interaction (Bausek *et al.* 2004; Stewart *et al.* 2004). ZP3 gene has been sequenced of 3,634 nucleotides and 9 exons coding for a protein 446 amino acids long and comparative study been done by 17 different avian species (Calkins *et al.* 2007). The concentration of calcium involves in inhibition of spermatozoa motility and zinc depress turkey sperm metabolism (Holm *et al.* 2000). Phosphatidylinositol 3-Kinase regulating calcium flagellar movement of fowl spermatozoa (Ashizawa *et al.* 2009).

4. Primordial germ cells (PGCs) migration

4.1. Primordial germ cells (PGCs)

Primordial germ cells (PGCs) are precursors of functional gametes and segregated from somatic cell lineages in the initial developmental phase. It is originated from the epiblast in stage X embryo and then translocate into hypoblast.

The theories of PGC differentiation occur to two different mechanisms which are preformation and induction (Extavour & Akam 2003). Several researchers report that the PGCs from germinal crescent in stage 4 to 10 (Goldsmith 1935; Willier 1937; Reynaud 1969; Wentworth *et al.* 1989).

The germline cell is called PGC in various species (Saitou & Yamaji 2010). The migration of PGC is actived to exploit and colonizes the developing gonads (Richardson & Lehmann 2010). The PGCs differentiate after settling down in gonads and go through spermatogenesis or oogenesis. PGCs show very unique characteristics in development and generate functional gametes.

4.2. The uses of PGCs to produce germline chimera

The first of germline chimera production was reported by Reynaud in 1976. PGCs taken from germinal crescent into recipient embryonic gonad was produced germline chimera (Reynaud 1976). It is improved, germline transmission rate to 14% used by injecting germinal crescent PGCs into the busulfan-sterilized embryo in 1993 (Vick *et al.* 1993).

Ficoll density gradient centrifugation methods have been successfully

produced germline chimeras (Tajima *et al.* 1993; Ono *et al.* 1998; Naito *et al.* 1999). The efficient of germline chimera transmission were very high in 47-97% (Naito *et al.* 1994b).

4.3. PGC isolation

PGCs are entering the blood vessel when it starts cardiac propulsion and blood circulation at the stage of 12 (Weiss & Andres 1952). The circulating blood peaked during stages 13 through 15 (Singh & Meyer 1967). The isolation of blood PGCs injected in to other poultry (Simkiss *et al.* 1989).

The gonadal PGCs (gPGCs) isolated from 5.5-day have migration activity which had passed the migration stage by Chang (Chang *et al.* 1997). It has succeeded long-term culture of gPGCs by Han (Han *et al.* 2002). It has improved more than 45% germline transmission rate used by 10-day-cultured gPGCs in 2003 (Park *et al.* 2003).

4.4. PGCs migration

PGCs migrated to gonadal anlagen via bloodstream which is anterior region of germinal crescent (Hamburger & Hamilton 1951). The migration of PGCs early segregation, it is possible to perceive that unique migration pathway from PGC.

The utilized signaling pathway-related and adhesion-related molecules are associated the destination of PGC migration (Richardson & Lehmann 2010). PGCs are entering blood vessels at HH stage 10-12 in chicken. It is circulating until settling down to the genital ridge in avian species (Hamburger & Hamilton 1951; Ukeshima & Fujimoto 1991).

Three major migratory routes been established before PGCs have reached gonad and developing in chicken. Initially, PGCs migrate from central region of the area pellucida to the germinal crescent at HH stage X to 4 (Hamburger & Hamilton 1951; Eyal-Giladi & Kochav 1976; Ginsburg & Eyal-Giladi 1986), PGCs start to circulate in blood vessels during HH stages 12–15 (Fujimoto *et al.* 1976). Finally, PGCs settle down in the genital ridges at HH stage 17 (Fujimoto *et al.* 1976).

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CHAPTER 3

Improvement Of Survival Rate And Hatchability In Simplified Quail Egg Surrogate System For Single Cell Stage Of Embryo Development.

1. Introduction

An avian surrogate system is using recipient (surrogate) shell for the culture of an egg or embryo that was taken from a donor animal or freshly laid egg.

The environment for avian embryonic development is different than mammalian embryonic development. For instances, avian embryo grows in an egg outside of the body; the nutrition for developing embryo is supported by yolk deposited within the egg; an air space is located within the egg for the growth of embryo; and a thick egg shell protect the developing embryo including yolk.

This egg shell provides an optimum shelter for the growth of embryo from another avian species**.** There were many studies demonstrates avian surrogate shell system for ex ovo culture**.** The surrogate egg shell system for the culture of avian embryos from single cell stage to hatch was developed first in 1988 (Perry, 1988a). Then, this technique has been applied to various studies in avian species, including embryo development, transgenic animal production, and tissue development (Byun et al., 2011; LaRue et al., 2003; Ono and Wakasugi, 1983).

More specifically, surrogate egg shell has been used for embryo culture system, embryo formation, xenotransplantation, tissue interaction, transgene, and many other experimental fields (Brand-Saberi et al., 1996; Diez-Fraile et al., 2010; Guinazu et al., 2007; Ono et al., 1994; Perry, 1988b)**.** In some studies, avian embryo culture in surrogate egg shell was replaced by artificial vessel (Kamihira et al., 1998).

Although there have been many applied studies with surrogate egg shell

system, the practical use of this system has not been sufficiently succeeded because of several limitations in technical adaptation. The hatchability of birds after incubation with surrogate egg shell system is very low that hindering further application. Several groups reported that surrogate system for the culture of single cell stage embryos produced low hatchability rate (Perry, 1988b; Tomao Ono, 1996). Therefore, several researchers have been studied with various experimental trials to improve the hatchability rate. Those trials include different surrogate vessel, different albumin pH calcium component, and different order of bird's shell (Kamihira et al., 1998; Ono et al., 2005; Tomao Ono, 1996).

It is important to understand the fertilization processes of avian embryos before culture of them in surrogate egg shell system. Avian fertilization is usually takes place in the infundibulum after ovulation (Olsen and Neher, 1948). The first cleavage is started around 4.5 h after ovulation (Hamburger and Hamilton, 1992). The embryo composed of around 50,000 – 60,000 cells at the time of oviposition (Spratt and Haas, 1961). Thus, avian surrogate egg shell system can be applicable to culture the embryos at all stages of embryos.

The main purpose of this study was to make higher percentage of hatchability in surrogate egg shell system. We have modified surrogate egg shell system for the culture of quail embryos at single-cell stage and from fertilized-fresh laid eggs. We found that many embryos were dead at a time of hatching, because of not able to come out from the egg shell (Ono et al., 2005; Perry, 1988b).

However, we do not clearly understood the reason for the death of embryos at the time of hatching. We suggest that omphalo-mesenteric vein operation may needed during hatching day in a surrogate system.

2. Materials and methods

Experimental animals

Birds

The care and experimental use of Japanese quails (*Coturnix japonica*) were approved by the Institute of Laboratory Animal Resources of Seoul National University (SNU-070823-5), Korea. Quails were maintained according to a standard management program at the University Animal Farm, Seoul National University. Animal management and reproduction were performed in accordance with standard protocols of Lab of Animal Genetic Engineering Laboratory, Seoul National University.

Collection of embryos

The egg laying time of quails was recorded by a video camera recorder system (Samsung Real Time DVR, SHR-6042, Seoul, Korea) to estimate the ovulation time. Female birds were sacrificed 70 – 120 min after oviposition and fertilized eggs with thick albumin were isolated. Normal laid eggs (Eyal-Giladi and Kochav stage X) was used as a control surrogate system.

Surrogate egg shell incubation system for ovipositioned eggs

Thin and thick albumen were collected from freshly laid quail eggs, and pH (7.4) and temperature (41.5℃) were adjusted within the CO2 incubator. The quail egg shell was prepared according to Ono & Wakasugi's method (1983). The

surrogate egg shells were wiped with 70% ethanol prior to manipulations. The narrow end of the surrogate shells (quail eggs, 12g of weight) was cut up to 20 mm in diameter to remove the egg contents.

The egg shell of quail egg weighing 14 g has been used for Q2 system because of the narrow end has been moved for incubation. Thus, the egg has been lost around 1.5 to 2 g of albumin. The blastoderm from freshly laid egg was gently transferred into a surrogate shell, and filled with thick albumen without air gap. Then, the surrogate shell was sealed by a plastic wrap, and fixed into a square frame as shown in the figure (Fig. 1a). A Showa egg incubator (Showa P008B-BIO, Showa Franky, Saitama, Japan) was used for egg incubation. The culture setup was operated for 2 days at 90 degree angle turning every 30 min with rocking around the long axis. After 2 days, all embryos and albumin were changed to thin with air gap (Q3 system), and the culture setup was operated at 30 degree angle turning every 30 min interval in vertical axis. Thus between Q2 and Q3 system were not changed surrogate egg shell.

Surrogate egg shell incubation system for single cell stage embryos

Thick albumen was collected from freshly laid quail eggs, and pH and temperature were adjusted as mentioned above within the CO2 incubator. The narrow end of the surrogate shells (quail eggs, 14 g of weight) was cut up to 20 mm in diameter to remove the egg contents.

The single-cell stage embryo was collected from the infundibulum or

upper part of magnum. The embryo along with small amount of thick albumen was transferred into a surrogate shell. Then, the surrogate shell was completely filled with thick albumen without air gap. The eggs were incubated in an incubation rotator system (Rotator RT-5, Taitec, Saitama-ken, Japan) for 22 h maintaining 41.5℃ temperature and 5% of CO2 with rotating speed of 1min 45 sec for each circle (Fig. 1b). After Q1 incubation, surrogate shells were gently moved from CO2 incubator. Thick albumin in the surrogate shells was removed using a 10 ml plastic syringe, and replaced by thin albumin without disturbing the embryos with air gap. Then, the eggs were transferred for Q2 incubation in the Showa incubator. The culture setup was operated for 2 days at 37.5℃ and 70% RH with rocking at 90 degree angle turning every 30 min around the long axis. The culture setup was operated at 37.5℃ and 70% RH with rocking at 30 degree angle turning every 30 min in vertical axis.

Various trials on surrogate egg shell incubation system

We performed six trials (systems A – F) on surrogate egg shell incubation system to examine the rate of hatchability. System A: The surrogate shell was filled with thick albumen of Japanese quail along with albumin capsulated blastoderm. After 2 days of incubation, the thick albumin was replaced with thin albumin. Finally, omphalo-mesenteric vein was operated as described below (Table 1). System B: The surrogate shell was filled with thick albumen of Japanese quail along with non-capsulated blastoderm. After 2 days of incubation, the thick albumin was replaced with thin albumin. Finally, omphalo-mesenteric vein was

operated (Table 1). System C: The surrogate shell was filled with thin albumen of Japanese quail along with capsulated blastoderm. The albumin was not changed until hatching (Table 1). System D: The surrogate shell was filled with thin albumen of Japanese quail along with non-capsulated blastoderm. The albumin was not changed until hatching (Table 1). System E: The surrogate shell was filled with all albumin and blastoderm of donor egg (Table 1). System F: Normal egg incubation (Freshly laid eggs) (Table 1).

Omphalomesenteric vein operation

Omphalo-mesentric vein operation was performed to help hatching of quails in the surrogate system. In this method, the eggs were stop rocking 1 - 2 days before expected day of hatch. Then, 10 - 15 small holes were made in the plastic wrap with a pin when the beak of embryos came out from the chorioallantoic membrane. The plastic wrap was replaced with a lid of plastic petri dish when the chorioallantoic membrane was dried. After dried, the chorioallantoic membrane was removed carefully without damage on the blood veins of quails. When the outside of omphalo-mesenteric vein was dried, the embryo was moved from the surrogate shell, and when the omphalo-mesenteric vein was dried completely, the vein was cut using micro scissors (micro scissors HB7383, HEBU, Tuttlingen Germany). An antiseptic (povidone-iodine) was used for navel clean to avoid any germ infection.

Statistical Analysis

Data was used for statistical analysis and standard deviation (SD)

calculation. Statistical analysis was performed to determine significant differences existed in the measured data by using the general linear model (PROC-GLM) of SAS software (SAS Institute, Inc. USA), and the least-significant difference (LSD) method. A P-value of less than 0.05 was considered to be statistically significant.

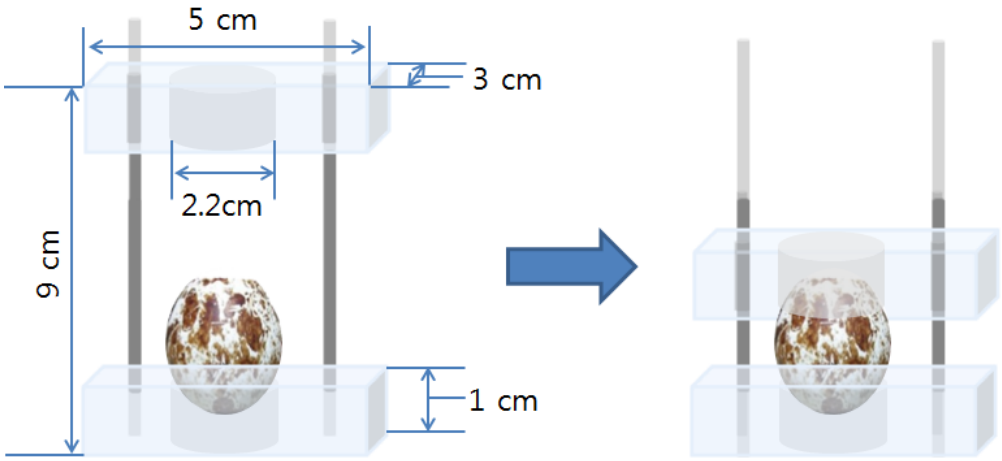
Graphical data

Graphical data on growth performances were prepared using a GraphPad (Prism 5) software. Viability and survival rate of quails in the surrogate egg shell incubation system were observed daily, and normal egg incubation was used as control.

3. Results and discussion

Traditionally, a pair of plastic rings was used for surrogate egg shell incubation system (Borwompinyo et al., 2005; Kamihira et al., 1998; Liu et al., 2012). Many researchers used this method for several years, and this system gave limited success on the hatchability of birds in surrogate incubation system (Ono et al., 1994; Tomao Ono, 1996). However, transferring single cell stage embryos needs fast manipulation process in order to avoid any effect by temperature and time consuming for single cell stage quail egg surrogate system. Therefore, we created square frame apparatus (Fig. 1a). In this study, this system was worked successfully on surrogate egg shell system, and it greatly reduced the manipulation time. In previous studies the viability of single cell embryos cultured in surrogate system was decreased at 0 – 6 days of incubation (Ono et al., 2005; Tomao Ono, 1996). In our study, the viability of single cell embryos was not decreased at 0 – 6 days of incubation. This result clearly suggests the importance of fast manipulation in surrogate incubation system. In addition, transferring egg shell from Q1 to Q2 system may cause damage on growing embryos. In our method, we could simply transfer the square frame apparatus that consists surrogate shell and growing embryos from Q1 to Q3 system without damaging the embryos and the process could be fast here.

a



b

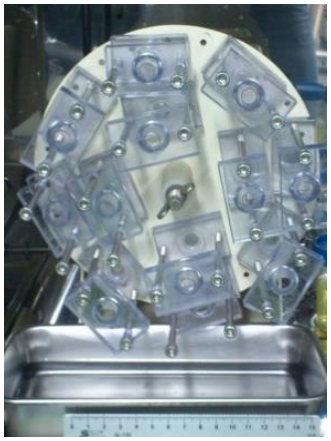


Figure 1. Surrogate culture systems. a, Square frame apparatus for the incubation of quail surrogate shell. A surrogate shell from Japanese quail egg was prepared for the incubation of donor quail embryos. After filling with embryo and albumin, the surrogate shell was sealed with plastic wrap and square frame apparatus. b, The incubation rotator system, which was operated for 22 h at 41.5℃ and 5% of CO2 with 1 min 45 sec of rounding speed for each circle.

We performed six different trials on surrogate egg shell incubation in

order to improve the viability and hatchability that are, with and without albumin capsulated blastoderms, and with and without final omphalo-mesenteric vein operation. Figure 2 shows the rate of viability and hatchability of quail embryos grown in different trials of surrogate egg shell incubation system. The viability of embryos in surrogate egg shells with thick albumin capsulated (system A) was higher than that of non-capsulated (system B). There are two critical time points, incubation day 3 – 5 and close to hatching day, which affect the survival rate of embryos in surrogate egg shell incubation system, but the major reason is not known to our knowledge. Nevertheless there was dramatic changed in albumin pH between days 3 to 5 (Tomao Ono, 1996). This previous results show that clue of embryo mortality increase in those days. A few previous studies also reported that many birds were died at the time of hatching as well.

It was survived until hatching in system A and B with omphalo-mesentric vein operation. Thus, optimization of egg shell surrogate system and embryo manipulation particularly at hatch is necessary. We have compared the viability and hatchability between thick albumin capsulated and non-capsulated incubation systems (between systems A and B), and between thin albumin capsulated and non-capsulated incubation systems without changed albumin (between systems C, D and E). Results of these comparisons showed approximately 20 percentage of difference on survival rate (Fig. 2).

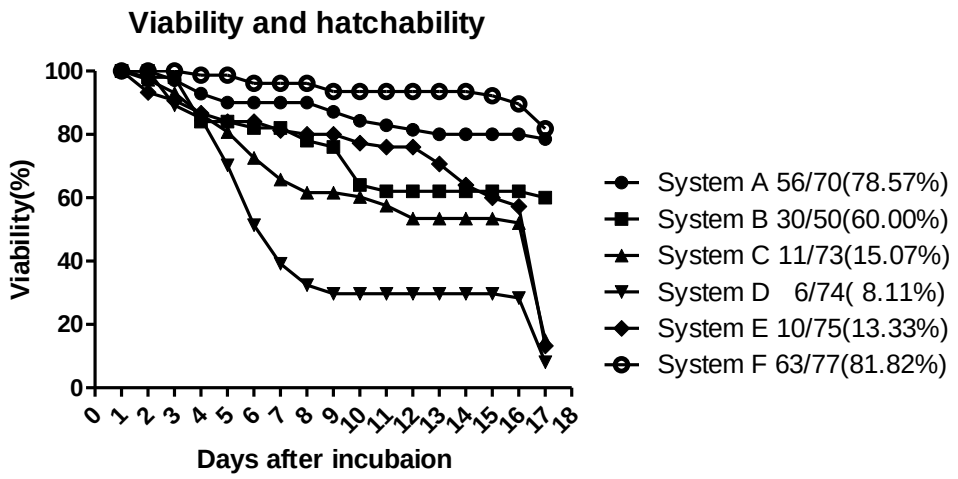


Figure 2. The comparative analysis of viability and hatchability in various trials of surrogate egg shell incubation system. System A: The surrogate shell was filled with thick albumen capsulated blasoderm. After 2 days of incubation, the thick albumin was replaced with thin albumin, and finally, omphalo-mesenteric vein was operated. System B: The surrogate shell was filled with thick albumen non-capsulated blastoderm. After 2 days of incubation, the thick albumin was replaced with thin albumin, and finally, omphalo-mesenteric vein was operated. System C: The surrogate shell was filled with thin albumen along with capsulated blastoderm. System D: The surrogate shell was filled with thin albumen along with non-capsulated blastoderm. System E: The surrogate shell was filled with all albumin and blastoderm of donor egg. System F: Normal egg incubation (control).

A higher percentage of hatchability was seen in system A followed by system B, because these two systems were subjected for omphalo-mesenteric vein operation at the time of hatching. As a consequence of the percentage of hatchability was low in systems C, D, and E lack of omphalo-mesenteric vein operation. Moreover, the thin albumin was not changed in systems C, D, and E during the incubation time. Therefore, we suggest that changing thin albumin during incubation and omphalo-mesenteric vein operation at hatching is crucial for the improvement of viability and hatchability in surrogate egg shell incubation system. In a different experiment, we cultured the single cell stage embryos in surrogate egg shell incubation system with slight modifications of Ono's method (Ono and Wakasugi, 1983). We obtained approximately 60% hatchability from single cell stage embryos cultured in surrogate egg shell system (Fig. 3). There are some critical times in the culture of single cell stage embryos also. Particularly, the incubation days between 2 to 3 affects the survival rate. The reason here may be due to replacement of thick albumin with thin albumin (Table 1). The percentage of hatch from single cell stage embryos in egg shell surrogate system is sufficient, and may be applicable for genetic manipulation study.

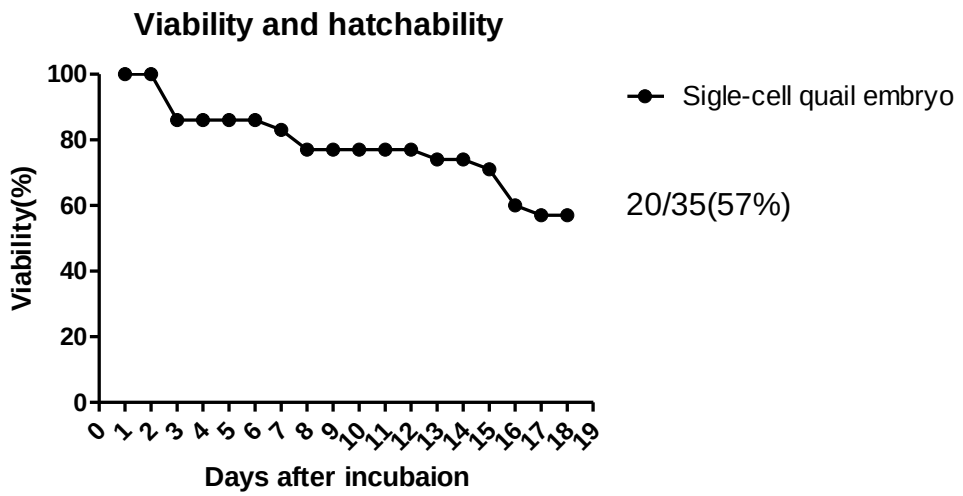


Figure 3. The viability and hatchability analysis of single cell quail embryos incubated in surrogate egg shell system 1 with omphalo-mesenteric vein operation.

Table 1. Systems for the culture of embryos from the blastoderm stage to hatching

System	Type of embryo culture	Days of incubation		
		1	2	3 to 17
A	Incubation albumin	Thick albumin	Thin albumin	Thin albumin
	Used surrogate egg weight(g)		14	
	Diameter of shell opening (mm)	20		20
	Omphalo-mesenteric vein operation			O
	Thick albumin capsulated	Attached		
B	Incubation albumin	Thick albumin	Thin albumin	Thin albumin
	Used surrogate egg weight(g)		14	
	Diameter of shell opening (mm)	20		20
	Omphalo-mesenteric vein operation			O
	Thick albumin capsulated	Removed		
C	Incubation albumin	Thin albumin		Thin albumin
	Used surrogate egg weight(g)		14	
	Diameter of shell opening (mm)	20		20
	Omphalo-mesenteric vein operation			X
	Thick albumin capsulated	Attached		
D	Incubation albumin	Thin albumin		Thin albumin
	Used surrogate egg weight(g)		14	
	Diameter of shell opening (mm)	20		20
	Omphalo-mesenteric vein operation			X
	Thick albumin capsulated	Removed		
E	Incubation albumin	All transfer(thick and thin)		
	Used surrogate egg weight(g)		14	
	Diameter of shell opening (mm)	20		20
	Omphalo-mesenteric vein operation			X
	Thick albumin capsulated	Attached		
F	Incubation albumin	Natural incubation		
	Used surrogate egg weight(g)		NA	
	Diameter of shell opening (mm)			

Omphalo-mesenteric vein operation	X
Thick albumin capsulated	

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CHAPTER 4

Fertilization by Intracytoplasmic Sperm Injection (ICSI) of Cryopreserved Sperm into Unfertilized Quail Ovum

1. Introduction

Conservation of avian genetic resources through sperm cryopreservation has been studied for more than 80 years. In 1941, Shaffner reported fertilization of chicken eggs by artificial insemination with cryopreserved semen, but no chick hatched (Shaffner et al., 1941). Polge found that glycerol could be used as a cryoprotectant for avian sperm cryopreservation (Polge, 1951). To date, many protocols have been established for sperm cryopreservation (Bellagamba F, 1993; Donoghuea and Wishart, 2000; Lake, 1986; Surai PF, 1996). Although fertility rate after artificial insemination with cryopreserved sperm has increased to 30-60% in several avian species such as chicken, turkey and gander, low fertility rate in other birds is still challenging (Blesbois, 2007). Also, hatchability with frozen-thawed sperms is still too low (less than 2%) to be applied to commercial application, which should be overcome in the further study (Blesbois, 2007).

Since intracytoplasmic sperm injection (ICSI) had been developed in many animal species, as well as humans, this technique has also been established with a high rate of fertilization in avian species (Hrabia et al., 2003; Mizushima et al., 2007; Obruca et al., 1995; Sato et al., 1999; Takagi et al., 2007; Yanagimachi, 2005). ICSI system could be utilized not only to conserve endangered species but also to produce transgenic birds. In addition, the oocyte activation by ICSI can give useful information for investigating the processes and mechanisms of early embryonic development in birds. Because the cryopreserved sperm usually lack mobility which causes reduced fertility after artificial insemination as studied in chicken and turkey (Bakst and Sexton, 1979; Scott et al., 1980; Westfall and Harris, 1975), it is hypothesized that direct injection of cryopreserved sperm into the oocytes can increase the proper embryo development rate.

Avian embryo culture systems with a surrogate eggshell have been used since 1984 (Ono and Wakasugi, 1984) and a complete culture system from a fertilized ovum to a hatched chick was established (Perry, 1988). This incubation

system has been expanded to include studies on avian embryo development, transgenic animal production, and tissue development (Byun et al., 2011; LaRue et al., 2003; Ono and Wakasugi, 1984). In this study, ICSI with cryopreserved sperm and following surrogate eggshell culture was conducted to develop an integrated system for preservation of avian genetic resources.

2. Materials and methods

2.1. Experimental animals

Japanese quails (*Coturnix japonica*) were managed according to our standard operation protocols, and all experimental procedures were approved by the Institutional Animal Care and Use Committee, Seoul National University, before experiments were performed (SNU-070823-5).

2.2. Collection of unfertilized oocytes and semen

Egg-laying time for each quail was recorded using a video recording system (SHR-6042 Real Time DVR; Samsung, Seoul, Korea) to estimate the ovulation time, and oocytes were collected according to the previous study with minor modification (Hrabia et al., 2003). Briefly, to collect the freshly ovulated oocytes prior to the degeneration process, egg-laying time was monitored using a video recording system (Fig. 1A). Quails were sacrificed 70–120 min after previous egg laying, and the freshly ovulated oocytes were retrieved surgically from the infundibulum or the upper part of the magnum (Fig. 1B).

2.3. Collection and preparation of ejaculated sperm for ICSI

The sperm preparation method for ICSI was adopted from Mizushima *et al*. (Mizushima S., 2008). Quail semen for ICSI was obtained from adult male quails by ejaculation via conventional lumbar massage (Fig. 1C). The ejaculated semen was washed with DMEM three times by gentle centrifugation to remove other semen components.

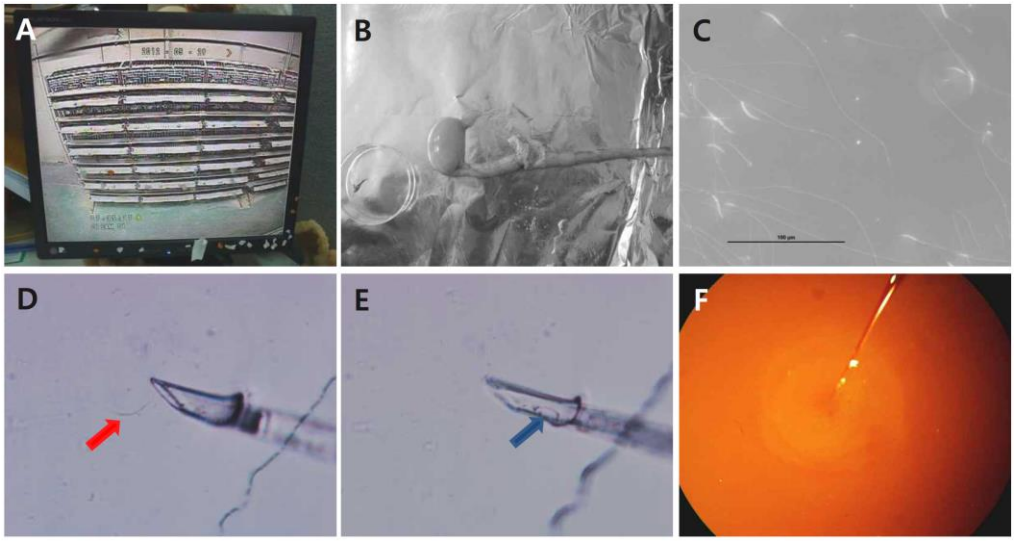

Figure 1. Oocyte collection and intracytoplasmic sperm injection (ICSI) in quail. (**A**) To predict the ovulation time, egg-laying time of each quail was recorded by using recording system. (**B**) Quails were sacrificed 70–120 min after oviposition, and the oocytes were recovered from the infundibulum or the upper part of the magnum. (**C**) Collected quail semen including sperm (**D**-**E**) A single sperm was picked up into an injection micropipette (**D**; red arrow, before pick-up, **E**; blue arrow, after pick-up). (**F**) Collected sperm was injected into the oocyte by the micro-injector under the microscope.

2.4. Semen freezing and thawing protocols

The method for semen freezing and thawing was modified from Lake *et al*. (Lake et al., 1981). Pooled semen was collected in a total volume of 1:1 (v:v) Lake Freezing diluents containing cryoprotectants (glycerol) with other materials as previously described (Lake et al., 1981). To optimize the cryopreservation condition, sperm motility was tested with two cryoprotectants (glycerol and dimethylacetamide (DMA)) by combinations of different ratio between them. As shown in Table 1, 11% of cryoprotectants had higher motility than other groups. And the combination of 8% glycerol and 3% DMA was the highest motility (48%), so that this combination was used in this study.

The diluted sperm was gently mixed, and equilibrated for 20 min in 4℃. The straws were placed in liquid nitrogen with 5 cm plastic bout and frozen for 20 min, and then plunged into liquid nitrogen. The straws thawed water bath in 4℃ and quickly opened. The semen transferred to 1.5 ml tube and was diluted with thawing diluents (pre-freezing diluent), and glycerol was removed by centrifugation. Finally, the semen pellet was resolved in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 4 mg/mL of bovine serum albumin (BSA, fraction V; Sigma-Aldrich). To collect sperm for injection, 1 μL of DMEM containing the sperm was placed on a Petri dish covered with mineral oil.

2.5. Synthesis of quail PLCζ cRNA and preparation of IP_3

The quail PLCζ complementary DNA (cDNA) was synthesized by reverse transcription-polymerase chain reaction (RT-PCR) with total RNA extracted from testis as described by Mizushima *et al*. (Mizushima et al., 2009). The PLCζ cDNA fragment was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and then PLCζ cDNA was linearized by digestion with *SpeI* restriction enzyme. Finally, PLCζ cRNA was synthesized using the TranscriptAid

T7 High Yield Transcription Kit (Fermentas, Loughborough, Leicestershire, UK) according to the manufacturer's instructions. 6 μg/mL of D-*myo*-inositol 1,4,5-trisphosphate trisodium salt (IP_3) (Sigma-Aldrich) diluted in distilled water was used for co-injection with sperm.

2.6. Microinjection and surrogate eggshell culture

ICSI was performed according to Hrabia *et al*. (Hrabia et al., 2003). Collected oocytes were placed in DMEM, and the ejaculated or cryopreserved/thawed sperm were identified under a Hoffman modulation contrast microscope (Eclipse Ti-U; Nikon, Tokyo, Japan). Subsequently, the sperm were injected immediately into the central area of the germinal disc using a micromanipulator connected to a micro-injector (IM-9B; Narishige Instruments, Tokyo, Japan) (Fig. 1D-F).

The surrogate eggshells were prepared by cutting along the sharp end, and the yolk and albumen were removed. After a small amount of thick albumen was added into the surrogate shells, the manipulated oocytes were placed inside. Finally, the surrogate shells were covered with a plastic wrap and sealed with plastic frame. The ICSI embryos in surrogate shells were incubated in a CO_2 incubator for 24 h at 41.5℃ and 10% CO_2 to mimic intrauterine egg development (Fig. 2A). Subsequently, the embryos were incubated at 38.5℃ and 70% humidity with rocking at a 90° angle and 30 min intervals until the next experiment. This method was modified from Ono and Wakasugi's surrogate methods (Ono and Wakasugi, 1984).

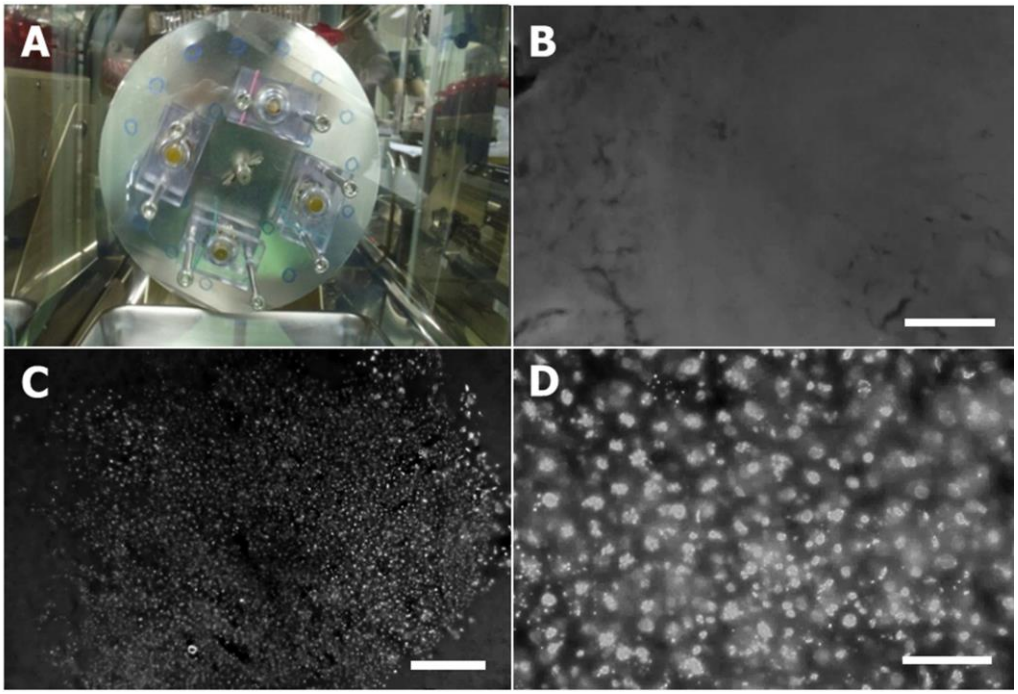

Figure 2. Surrogate eggshell culture system and blastoderm formation after ICSI. (**A**) After ICSI, manipulated oocytes were incubated in surrogate eggshells with gentle rotation for early embryo development. (**B**) In a control oocyte without ICSI, no DAPI-positive dividing cell was detected. (**C**) 24 h after ICSI most embryos developed to EGK stage VI-VII showing the morphological characteristics of the developing embryo. (**D**) The magnified view of (**C**). Scale bars = 500 µm in (**B and C**) and 100 µm in (**D**), respectively.

2.7. DAPI staining

After development stages of ICSI embryos were determined according to Eyal-Giladi and Kochav (EGK) stages (Eyal-Giladi and Kochav, 1976) and Hamburger and Hamilton (HH) stages (Hamburger and Hamilton, 1951), the embryos were fixed with 4% (w/v) paraformaldehyde in 1× phosphate-buffered saline (PBS) and then stained with 4′,6′-diamidino-2-phenylindole (DAPI). DAPI-stained embryos were observed under a fluorescent microscope (Eclipse Ti-U; Nikon) to confirm fertilization and status of embryonic development (Ainsworth et al., 2010; Hrabia et al., 2003).

2.8. Statistical analysis

Statistical analyses were performed using the Student t test in SAS version 9.3 software (SAS Institute, Cary, NC). The significance levels between control and treatment groups were analyzed using the general linear model (PROC-GLM) in SAS software. Differences between treatments were deemed to be significant when $P < 0.05$.

3. Results and Discussion

3.1. ICSI with freshly isolated sperm

To evaluate fertilization and embryo development after ICSI, fresh sperm were injected into the oocyte, and manipulated embryos were stained with DAPI, a nucleus-specific dye, after 24 h of culture, (Fig. 2). In the control oocytes without ICSI, DAPI-positive cells were not detected (Fig. 2B). After injection with fresh sperms, however, embryo development was detected showing many dividing nuclei (Fig. 2C and D). The ratio of blastoderm formation after ICSI with fresh sperm was approximately 13% (3/24) (Table 1). The ratio was similar to the results of that of Hrabia *et al.* (Hrabia et al., 2003) and Mizushima *et al*. (Mizushima S., 2008).

To increase embryo development after ICSI, PLCζ cRNA or IP_3, oocyte activation inducer (Mizushima S., 2008), was co-injected with the fresh sperms. Remarkably, co-injection of PLCζ cRNA or IP_3 showed significantly higher development efficiencies compared to injection with only fresh sperm (13% in controls *vs*. 36% with PLCζ cRNA and 90% with IP_3; Table 1). Previous studies have demonstrated that PLCζ cRNA and IP_3 can induce Ca^{2+} oscillation leading to oocyte activation and resume meiotic maturation in mammals and birds (Mizushima et al., 2007; Stricker, 1997, 1999), indicating that the injection of a single sperm without any oocyte-activating factors is insufficient for Ca^{2+} oscillation and following blastoderm formation. Therefore, oocyte activation inducers such as strontium treatment, PLCζ cRNA, and IP_3 are crucial to improve the fertility in ICSI embryos. In this regard, this system could be also utilized for the gene transfer to generate transgenic birds (Mizushima et al., 2010; Mizushima et al., 2009; Mizushima S., 2008; Shimada et al., 2014).

In the previous studies, ICSI together with PLCζ cRNA resulted in embryonic death before HH stages 6–7 (Mizushima et al., 2007, 2009). However, in this study, approximately 15% of embryos developed to over stage 19 (Table 2 and Fig. 3). In addition, using the ICSI combined with the surrogate system, one quail embryo developed to HH stage 39 (embryonic day 11; Fig. 3). These results indicate that surrogate culture system immediately after ICSI is more efficient for

normal embryonic development than the conventional plastic culture as described in the previous study (Hrabia et al., 2003). Since the first successful trial of ICSI in mammals by Uehara and Yanagimachi (Uehara and Yanagimachi, 1976), ICSI has been applied to many studies to produce offspring in mammalian species, including humans (Catt and Rhodes, 1995; Farstad, 2000; Kim et al., 1998; Palermo et al., 1992; Palermo et al., 1995). This systematic protocol including ICSI and the surrogate culture could be utilized for other applications such as transgenesis and avian genetic resource conservation.

Table 1. Embryo development 24 h after ICSI with fresh sperm, with or without PLCζ cRNA or IP3

Sample	No. of oocytes injected	No. of developing embryos (%)
Fresh sperm	24	3 (13%)
Fresh sperm with PLCζ cRNA	36	13 (36%)
Fresh sperm with IP3	20	18 (90%)

Table 2. Embryo development of quail oocytes after injection of fresh quail sperm with IP3 in surrogate egg shell incubation

Sample	No. of oocytes injected	No. of embryos survived beyond EGK stage X	No. of embryos developing to:						
Fresh sperm with IP3	65	17	HH stage	4	6	19	28	29	32	39
			No. of embryos	3	3	1	6	1	2	1

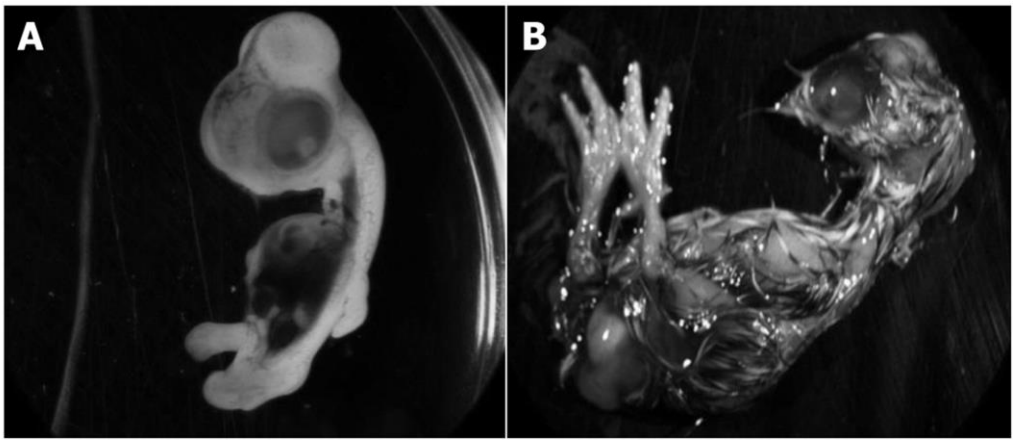

Figure 3. Quail embryo development after ICSI with fresh sperm. After ICSI, the manipulated embryos were further incubated over EGK stage X. Examples of embryos which developed to HH stage 29 (**A**) and HH stage 39 (**B**), respectively.

Table 3. The quail oocytes viability on surrogate incubation in 24 h after injection of thawed cryopreserved quail sperm

Sample	No. of oocytes injected	No. of developing embryos (%)
Cryopreserved/thawed sperm (control)	20	6 (30%)
Cryopreserved/thawed sperm with PLCζ cRNA	26	13 (50%)
Cryopreserved/thawed sperm with IP3	46	34 (74%)***

***$P < 0.001$ compared with the control

3.2. ICSI with cryopreserved and thawed sperm

In the next experiment, cryopreserved/thawed sperm were used for ICSI to investigate the possibility of the use of long-term preserved sperm for conservation of avian species (Fig. 4 and Table 3). Similar to the results from fresh sperms, the manipulated oocytes showed blastoderm formation 24 h after ICSI with cryopreserved/thawed sperms (Fig. 4). The embryo development ratio was approximately 30 % (6/20) (Table 3). In mammals, the ICSI system showed that cryopreserved sperm have higher fertility compared to normal fresh sperm injection (Cayan et al., 2001; Wald et al., 2006). Additionally, the efficiency was not significantly different between fresh and cryopreserved epididymal *versus* testicular spermatozoa (Desai et al., 2009).

Next, the cryopreserved sperms were co-injected with PLCζ cRNA or IP_3. Similar to the results with fresh sperm, PLCζ cRNA and IP_3 enhanced the oocyte activation and following blastoderm formation compared to ICSI with sperm only (30% with sperm only *vs*. 50% with PLCζ and 74% with IP_3). Nevertheless, the overall embryo development ratio of ICSI with the cryopreserved/thawed sperm was lower than that with the fresh sperm (Tables 2 and 3). In spite of developmental progress of the embryos after ICSI, none of them developed beyond EGK stage X. It seems that cryopreservation step damage to membrane of quail sperm and interfere with normal development of the oocytes. Similarly, in mice, the rate of blastocyst development of zygote produced by ICSI of membrane-damaged sperm was relatively lower compared with the rate obtained by fresh sperm (Perry et al., 1999). Another possible reason is harmfulness of cyroprotectant. Sperm cryopreservation methods have been developed in avian species using various types of cryoprotectants such as glycerol, DMA, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), ethylene glycol (EG), and *N*-methylacetamide (MA) (Chalah et al., 1999; Lee et al., 2012; Tselutin et al., 1995; Tselutin et al., 1999). Although researchers have focused on optimizing the methodology for avian sperm cryopreservation and offspring production

(Donoghuea and Wishart, 2000), it has been reported that several cryoprotectants can be harmful to sperm survival and fertilization (Tselutin et al., 1999).

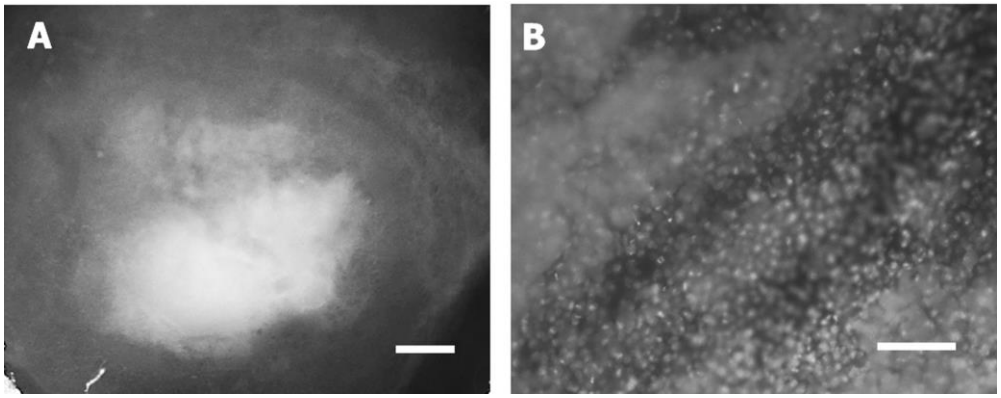

Figure 4. (**A**) A phase-contrast image of quail embryo which developed to EGK stage X after ICSI. (**B**) A fluorescent image of an ICSI embryo after DAPI staining to detect cell division. Scale bars = 500 µm and 100 µm in (A) and (B), respectively.

4. Conclusions

This study was conducted to develop the ICSI technique and surrogate system to activate fertilization and development of quail embryos by using cryopreserved quail sperm (Fig. 5). The cryopreserved-thawed sperm showed 30% of embryo development ratio after ICSI. In addition, when they were injected with IP_3, the embryo development ratio significantly increased to 74%. Because there was no hatched chick in this study, to utilize cryopreserved sperm with ICSI and surrogate eggshell system for conservation of avian species, methods for increasing viability and hatchability should be investigated in the further study.

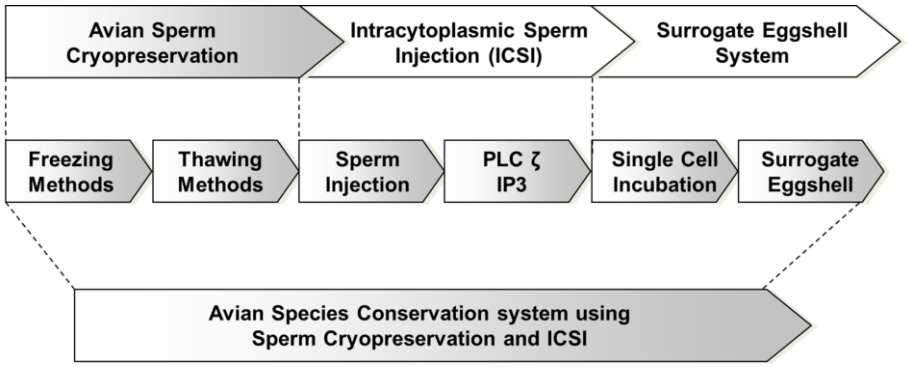

Figure 5. The systematic protocol including sperm cryopreservation, ICSI, and surrogate eggshell culture can be useful for conservation of avian genetic resources.

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CHAPTER 5

Spatial and Temporal Action of Chicken Primordial Germ Cells during Initial Migration

1. Introduction

In embryogenesis, cell migration plays a central role for the development and morphogenesis of organisms. During passive migration, cells are transffered by motile neighboring cells. But, cells acquire their active movement by a dynamic and integrated system including morphological polarization, membrane extension and change in migration speed governed by various molecular mechanisms (Lauffenburger & Horwitz 1996). In particular, spatially and temporally appropriate migration of cells in embryogenesis is closely related to their fate dicision. Thus, studies on cell migration mechanism help us to understand characteristics and differentiation of cells.

In most animals, primordial germ cells (PGCs), the precursors of functional gametes, are segregated from somatic cell lineages in the initial developmental phase, although they are specified by two different mechanisms in different species: preformation and induction (Extavour & Akam 2003). In addition to their early segregation, one of the conserved and striking features of PGCs is their unique migration pathway. During migration toward their destination, PGCs are regulated by intrinsic and extrinsic guidance mechanisms, which utilize signaling pathway-related and adhesion-related molecules (Richardson & Lehmann 2010). Because proper cell migration is crucial for normal embryo development and tissue differentiation (Franz *et al.* 2002), studying the migration mechanism of PGCs is also important for understanding the biology of other migrating cell types.

Chicken PGCs are normally located in the central region of the area pellucida which contains both the epiblast and hypoblast before primitive streak formation (Ginsburg & Eyal-Giladi 1987), and initiate their migration from this region. Chicken PGCs have three major migratory routes before they settle within the developing gonads. First, they migrate from the central region of the area pellucida at Eyal-Giladi and Kochav (EGK) stage X to the germinal crescent at Hamburger and Hamilton (HH) stage 4 (Eyal-Giladi & Kochav 1976; Ginsburg &

Eyal-Giladi 1986; Hamburger & Hamilton 1992). Second, they undergo circulation in extraembryonic blood vessels during HH stages 12–15 (Swift 1914; Fujimoto *et al.* 1976b). Finally, they settle down in the genital ridges at HH stage 17 (Fujimoto *et al.* 1976a). Several studies have uncovered the mechanisms by which the PGCs become lodged in the vascular system and colonize the gonads (Stebler *et al.* 2004; De Melo Bernardo *et al.* 2012). However, how they migrate toward the anterior region called the germinal crescent is less well studied.

To elucidate the mechanism of chicken PGC migration, we conducted spatial and temporal analyses during their first-phase migration from the central region of the area pellucida toward the germinal crescent. We investigated the migration route of fluorescenctly labeled exogenous PGCs that were transplanted into EGK stage X embryos and compared it to that of endogenous PGCs and somatic cells. Based on our results, we explain that passive and active forces sequentially regulate the migration of PGCs toward the germinal crescent.

2. Materials and methods

Experimental animals and animal care

White Leghorn chickens were managed according to our standard operation protocols, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University, before the experiments were performed (SNU-070823-5). Transgenic (TG) embryos expressing green fluorescent protein (GFP) under the control of the *CMV* promoter were derived in our previous study (Park & Han 2012).

Whole-mount in situ *hybridization*

To make hybridization probes, total RNA from magnetic-activated cell sorting (MACS)-positive PGCs at E6.5 was reverse-transcribed, and the resulting cDNA was amplified using chicken deleted in azoospermia-like (c*DAZL*)-specific primers (F, 5′-CGT CAA CAA CCT GCC AAG GA-3′ and R, 5′-TTC TTT GCT CCC CAG GAA CC-3′; product size 540 bp), as described previously (Rengaraj *et al.* 2010). Polymerase chain reaction (PCR) products of the correct size were cloned into the pGEM-T vector (Promega, Madison, WI, USA). After sequence verification, the recombinant plasmids containing the gene were amplified using T7- and SP6-specific primers (T7, 5′-TGT AAT ACG ACT CAC TAT AGG G-3′ and SP6, 5′-CTA TTT AGG TGA CAC TAT AGA AT-3′) to prepare the template for labeling the hybridization probes. Digoxigenin (Cordova *et al.*)-labeled sense and antisense c*DAZL* hybridization probes were transcribed *in vitro* using a DIG RNA labeling kit (Roche Diagnostics, Indianapolis, IN, USA). A standard protocol for whole-mount *in situ* hybridization in chickens was followed (Stern 1998).

Migration assay

In vitro cultured PGCs with or without GFP expression (Park & Han

2012) and DF-1 cells (a continuous chicken embryonic fibroblast cell line) were labeled with PKH26 fluorescent dye (Sigma-Aldrich, St. Louis, MO, USA) and then transferred into the subgerminal cavity or the posterior marginal zone (PMZ) of EGK stage X embryos. After sealing with Parafilm, eggs were further incubated at 37–38℃ under 60–70% relative humidity until HH stage 4 (18 h of incubation) or 10 (34 h of incubation) (Hamburger & Hamilton 1992). Embryos were detached from the yolk and transferred to saline to facilitate removal of the yolk and vitelline membrane. To assay migration into the gonads, eggs were incubated an additional 6 days until stage 28. Then, gonads and mesonephric kidneys were retrieved from the recipient embryos. The harvested embryos and gonads were fixed with 4% (w/v) paraformaldehyde in 1× phosphate-buffered saline (PBS) for further immunostaining or observed under an AZ100 multipurpose zoom confocal microscope (Nikon Corporation, Tokyo, Japan).

Whole-mount immunohistochemistry with cDAZL antibody

The polyclonal antibodies against cDAZL are produced by immunizing host rabbits with a synthetic peptide corresponding to residues near the N-terminus of cDAZL (SANAEAQCGSISEDNTH, amino acids 2-17). The specificity of cDAZL antibody on chicken PGCs was tested by co-immunostaining of *in vitro* cultured PGCs with anti- SSEA-1 and cDAZL antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (Supplementary Fig. S1). To label endogenous PGCs, the harvested whole-mount embryos were washed three times with PBS and blocked with blocking buffer (PBS containing 5% goat serum and 1% bovine serum albumin, BSA) for 1 h at room temperature. Samples were then incubated at 4°C overnight with rabbit anti-cDAZL. After washing with PBS three times, embryos were incubated with secondary antibodies labeled with fluorescein isothiocyanate (FITC; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 4 h at room temperature. The embryos were washed with PBS three times and visualized under the multipurpose zoom confocal microscope.

Time-lapse imaging with ex-ovo culture and migration assays

PKH26-labeled PGCs were transplanted into the subgerminal cavity of GFP_TG embryos at EGK stage X and *ex ovo* cultured for time-lapse imaging using a confocal microscope. The *ex ovo* culture was performed using an improved shell-less culture system (Yalcin *et al.* 2010). Briefly, cling wrap was affixed circumferentially on plastic cups containing warm sterile water using a rubber band to make a hammock. Embryos with yolks and albumin were placed onto the hammock. Then, a Petri dish was placed on top of the cup to seal the embryo. The embryo with the best morphology was selected and cultured in a Chamlide incubator (Live Cell Instrument, Seoul, Korea) at 37.8 ℃. Time-lapse images were taken using the AZ100 multipurpose zoom confocal microscope. To track selected cells, manual tracking was performed using NIS-Elements (Jia *et al.*) according to the manufacturer's. The centers of the manually selected cells were designated at each time point and the velocity of the cells was calculated based on the distance between their positions at two consecutive time points.

Statistical analysis

Statistical Analysis System software (SAS Institute, Cary, NC, USA) was used for analysis of all numerical data. Treatments were compared using the least-square difference or Duncan method, and analysis of variance (ANOVA) was used to determine the significance of the main effects. The level of significant differences was set at $P < 0.05$.

3. Results

Distribution of PGCs during primitive streak formation

To identify PGCs in whole embryos, *cDAZL* probes were used as a PGC specifc marker. At EGK stage XII (2 h of incubation), most of the PGCs were located in the central region. At HH stage 2 (6 h of incubation), some of PGCs exhibited anterior position. At HH stage 3 (12 h of incubation), many PGCs were gathered in the anterior region. At HH stage 5 (22 h of incubation), most of the PGCs were clearly located in the germinal crescent region (Fig. 1).

Passive migration of PGCs depends on their initial location

To investigate the migratory pathway of PGCs during their first-phase of migration, PKH26-labeled exogenous PGCs were transplanted into the subgerminal cavities of EGK stage X embryos (Fig. 2A). During incubation, exogenous PGCs migrated and became located in the marginal area of HH stage 4 embryos, mostly in the anterior region (Fig. 2B). When PKH26-labeled DF-1 cells were transplanted as a control, They migrated and became located in the extreme marginal area of the embryo at HH stage 4 (Fig. 2C). Thus, both exogenous PGCs and DF-1 cells migrated toward the anterior marginal zone when they were transplanted into the subgerminal cavities of EGK stage X embryos ($n > 4$ for both PGCs and DF-1 cells).

Next, to access the ability of PGCs to migrate toward the anterior region, PKH26-labeled cells were transplanted into the posterior end (posterior marginal zone, PMZ) of EGK stage X embryos (Fig. 2D). The embryos were further incubated until HH stage 10 to give PGCs more time to migrate toward the anterior region ($n > 4$). At HH stage 10, PGCs had not reached the anterior region, but they had migrated and were located in the posterior half of the embryo (Fig. 2E). Exogenous DF-1 cells showed a similar pattern to PGCs ($n > 4$; Fig. 2F).

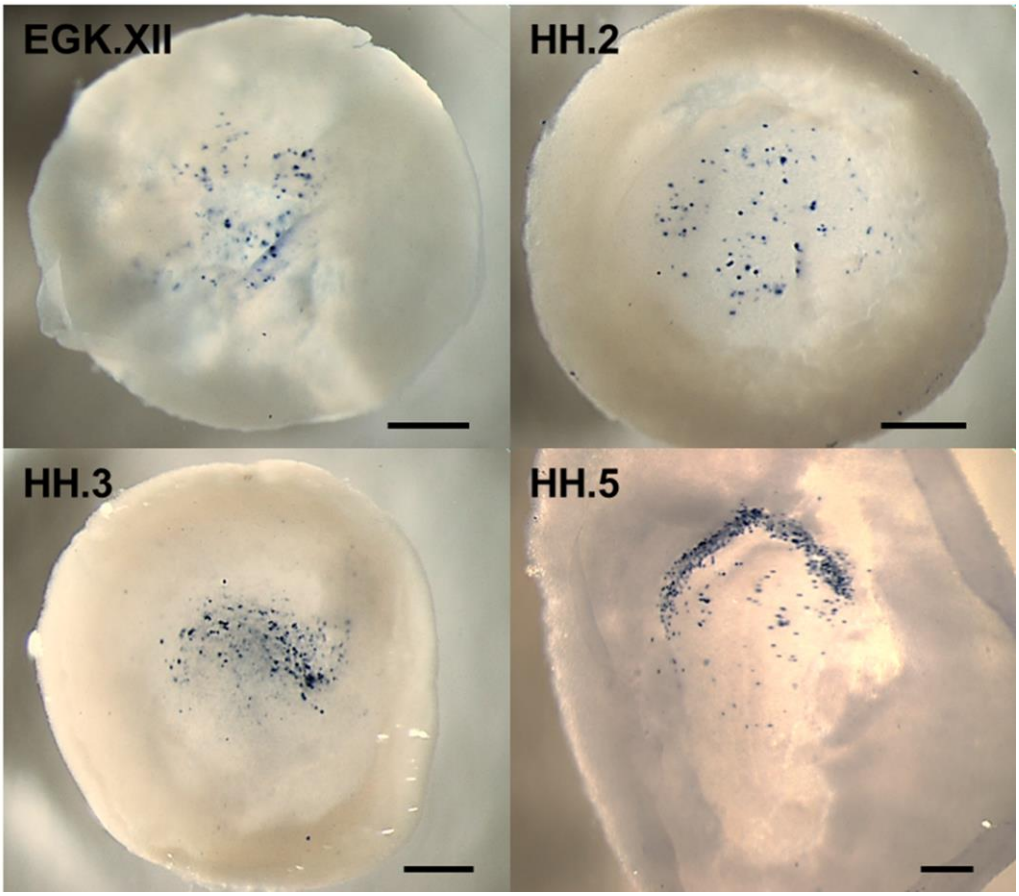

Figure 1. Distribution of PGCs during primitive streak formation. Whole embryos collected at EGK stage XII to HH stage 5 were hybridized with antisense probes for *cDAZL*. At HH stages 2 and 3, PGCs migrating toward the anterior region were observed. At HH stage 5, PGCs gathered in the germinal crescent were detected. The anteroposterior axis describes the areas above and below the area of primitive streak formation. Scale bars = 500 μm.

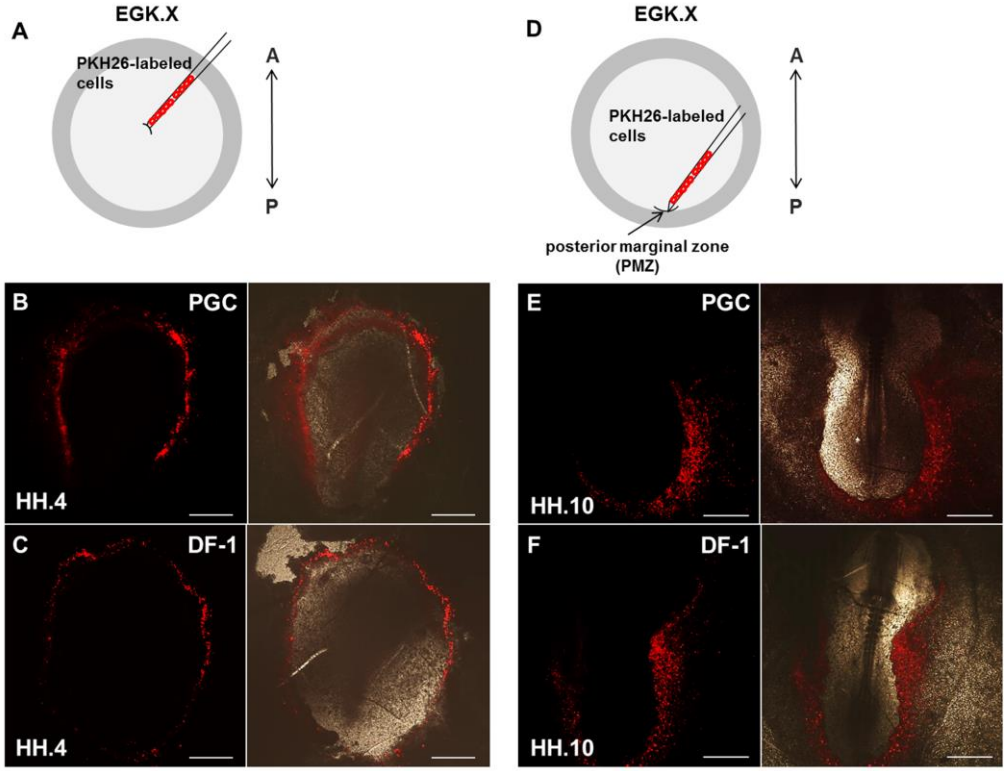

Figure 2. Effect of initial PGC location on passive migration toward the anterior marginal region. After PKH26 labeling, approximately 1,000 PGCs or DF-1 cells were transplanted into the subgerminal cavity **(A-C)** or the PMZ **(D-F)** in EGK stage X embryos. After incubation, the embryos were observed under a microscope at HH stage 4 or HH stage 10. When transplanted into the subgerminal cavity, both PGCs **(B)** and DF-1 cells **(C)** migrated toward the anterior region of embryos at HH stage 4. When transplanted into the PMZ, both PGCs **(E)** and DF-1 cells **(F)** reached the posterior half of the embryo. PMZ: posterior marginal zone. Scale bars = 1,000 μm.

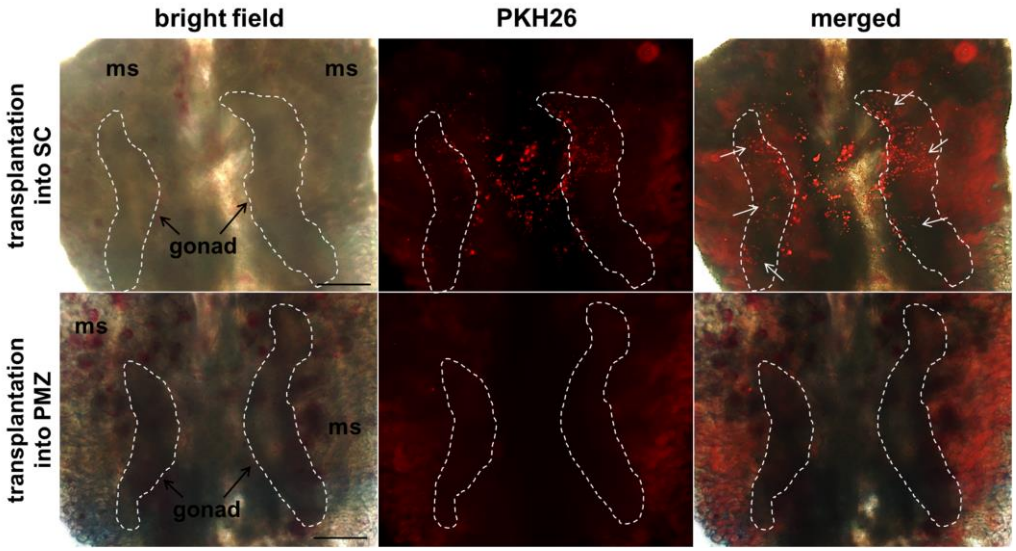

Figure 3. Effect of initial PGC location on migration toward the gonads. Approximately 5,000 PGCs were labeled with PKH26 and transplanted into the subgerminal cavity **(upper panels)** or the PMZ **(lower panels)** in EGK stage X embryos. After 6 days of incubation, the embryonic gonads and mesonephric kidneys (ms) were observed under a microscope. Migrating PGCs were only detected in the embryonic gonads (white arrows) when they were transplanted into the subgerminal cavity. The dotted lines indicate the embryonic gonads. PMZ: posterior marginal zone. Scale bars = 500 µm.

When the embryos were further incubated until embryonic day 6, PGCs transplanted into the subgerminal cavity migrated toward the embryonic gonads (n > 20). In contrast, PGCs transplanted into the PMZ did not migrate toward the gonads (Fig. 3). The above experiments confirm that PGCs transplanted into the subgerminal cavity could only migrate passively toward the anterior region of the embryo during hypoblast formation and contribute to germline development.

Active migration of PGCs toward the germinal crescent region

To clarify the ability of PGCs to migrate toward the germinal crescent region after injection into the subgerminal cavity, PKH26-labeled or GFP-expressing cell-transplanted embryos and non-transplanted embryos were incubated until HH stage 4. At HH stage 4, non-transplanted embryos were immunostained with anti-cDAZL antibody to identify endogenous PGCs and to compare their location with that of exogenous PGCs. Most of the exogenous PGCs were located in the germinal crescent region (Fig. 4A and C), as were most cDAZL-expressing endogenous PGCs (Fig. 4D). In contrast, most DF-1 cells were located outside of the germinal crescent region (Fig. 4B and C). Thus, exogenous PGCs and DF-1 cells were differently located in relation to the germinal crescent region, although both cell types migrated toward the anterior zone. In addition, cDAZL-expressing endogenous PGCs located laterally in the anterior region showed a polarized and amoeboid morphology, an indicator of active cell migration (Fig. 4D).

Active migration of PGCs toward the anterior head region before they enter the blood circulation

To investigate the ability of PGCs to migrate toward the anterior head region before they enter the circulation, PKH26-labeled or GFP-expressing cell-transplanted embryos and non-transplanted embryos were incubated until HH stage 10. At HH stage 10, non-transplanted embryos were immunostained with anti-

cDAZL antibody to identify endogenous PGCs and to compare their location with that of exogenous PGCs. Most of the exogenous PGCs were located in the anterior region of the area pellucida near the embryonic head (Fig. 4E and G). cDAZL-expressing endogenous PGCs were found in the same region (Fig. 4H). DF-1 cells were detected laterally in the area opaca, away from the anterior head (Fig. 4F and G).

Tracking PGCs between EGK stage X and HH stage 2 by time-lapse imaging

We confirmed that subgerminal cavity transplanted PGCs but not DF-1 cells can actively migrate into the germinal crescent region at HH stage 4 and incorporate into the anterior region of the area pellucida although both cell types passively migrate toward the anterior marginal region. To clarify the mechanism of PGC migration toward the germinal crescent region, time-lapse live imaging of the embryos for 8 h (EGK stage X to HH stage 2) was done after PKH26-labeled PGCs were transplanted into the subgerminal cavity of GFP-expressing TG embryos at EGK stage X (Fig. 5). The TG embryos express GFP ubiquitously under the control of the *CMV* promoter (Park & Han 2012). Then, the movements of several cells located in the centers of embryos were monitored. Some of the endogenous cells in the central axis at EGK stage X migrated in a straight line and reached the anterior region, while other endogenous cells located laterally migrated in a curved or circular line (Figs. 5A, D and G). These movements are identical to the normal migration of epiblast cells (Voiculescu *et al.* 2007). However, the PKH26-labeled exogenous PGCs located differently in the central regions of embryos

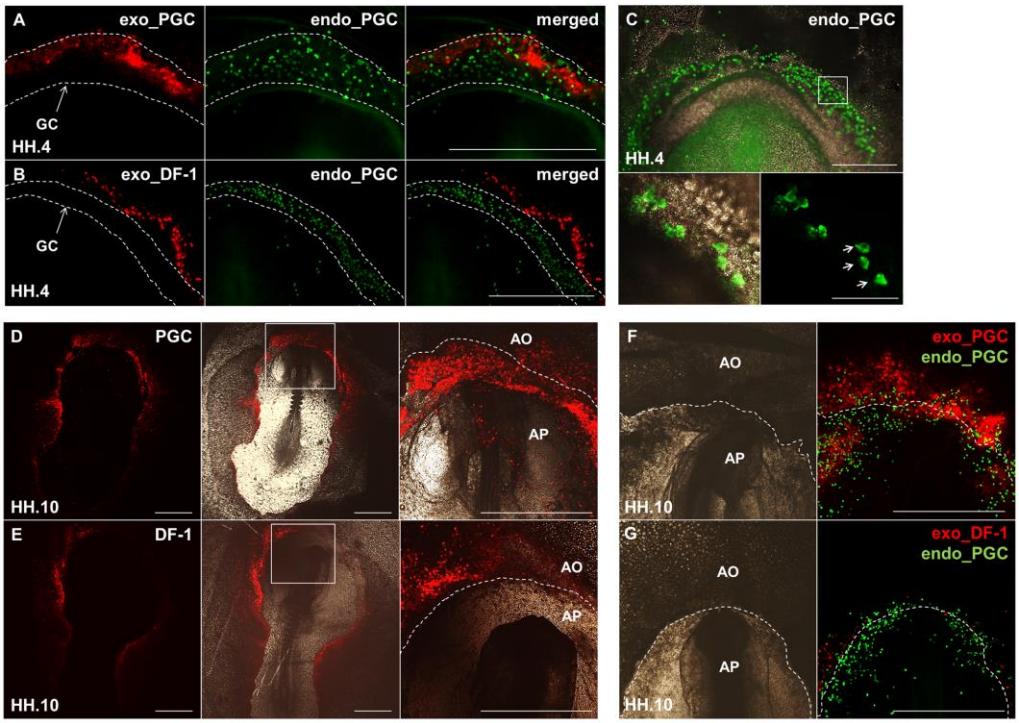

Figure 4. Differences in position between exogenous PGCs and DF-1 cells at HH stage 4 and 10. After PKH26-labeled PGCs, PKH26-labeled DF-1 cells, or both GFP_PGCs and PKH26-labeled DF-1 cells were transplanted into the subgerminal cavity in EGK stage X embryos, transplanted and non-transplanted embryos were incubated until HH stage 4 **(A-D)** and HH stage 10 **(E–H)**. After incubation, the non-transplanted embryos were immunostained with anti-cDAZL antibody **(D and H)** and observed under a microscope. At HH stage 4, PKH26-labeled or GFP-expressing exogenous PGCs and cDAZL-expressing endogenous PGCs were observed in the germinal crescent (GC, dotted lines) **(A, C, and D)**, while most exogenous DF-1 cells were not detected in the GC region **(B and C)**. White arrows in the magnified view **(D)** show the polarized and amoeboid morphology of endogenous PGCs. At HH stage 10, PKH26-labeled or GFP-expressing exogenous PGCs and cDAZL-expressing endogenous PGCs were located in the anterior region of the area pellucida (AP) near the embryonic head **(E, G, and H)**, while exogenous DF-1 cells were observed in the area opaca (AO) **(F**

and G). The dotted lines in **(E–H)** indicate the margin of the area opaca. Scale bars = 1,000 μm **(A–C and E–H)**, 500 μm **(D)** and 100 μm [magnified view of **(D)**].

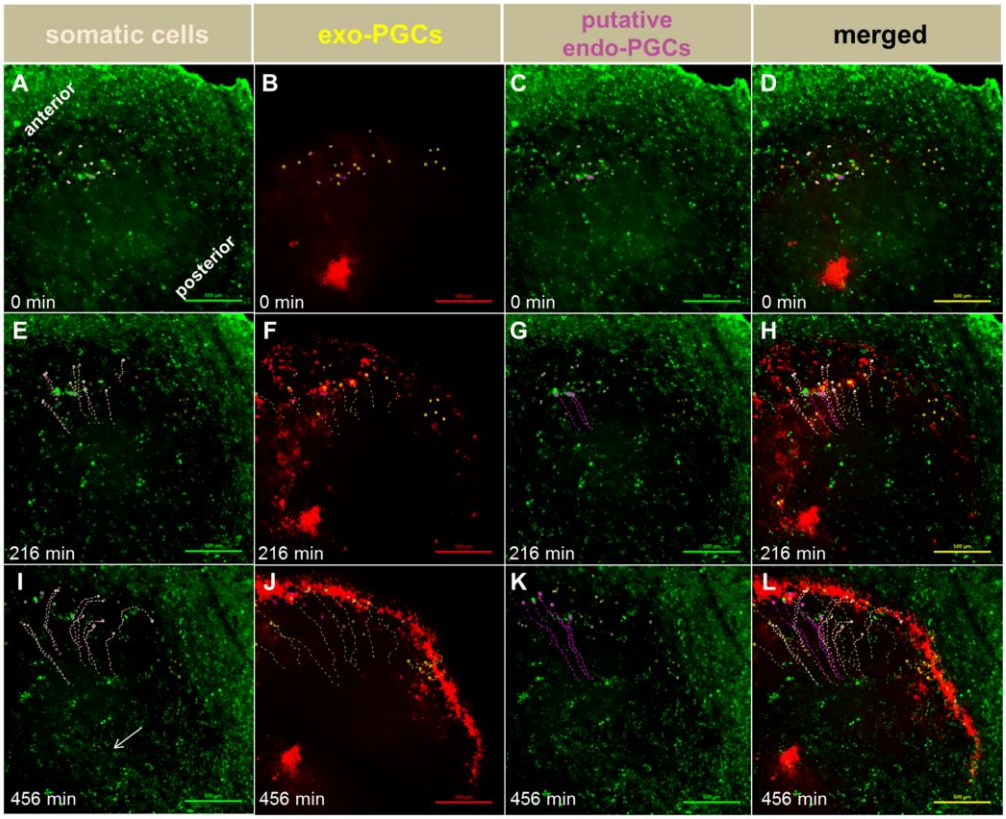

Figure 5. Migration pathway of PGCs between EGK stage X and HH stage 2. PKH26-labeled PGCs were transplanted into the subgerminal cavity in GFP-expressing transgenic embryos at EGK stage X. The embryos were then cultured in the live-imaging chamber for 8 h with time-lapse imaging using a confocal microscope. **(A, D, and G)** Tracking of somatic cells showing normal epiblast movement (white lines). **(B, E, and H)** Tracking of exogenous PGCs showing migration toward the anterior region (yellow lines). **(C, F, and I)** Merged images of all channels. For all images, the anteroposterior axis is from the upper left side to the lower right side. Scale bars = 500 μm.

migrated toward the anterior region in a straight line and settled in the future germinal crescent region at HH stage 2 (Figs. 5B, E and H). Thus, the exogenous PGCs showed apparent differences in migration pattern compared to somatic cells (Figs. 5C, F and I).

Since most of the traced cells showed anterior movement initially, changes in the migration speed of exogenous PGCs and somatic cells were investigated to clarify when and where active and passive forces of migration act (Fig. 6). During th initial 4 h of culture (EGK stages X–XII), all selected cells maintained a constant speed until they reached the anterior one third of the embryo. After 4 h, exogenous PGCs exhibited faster migration while the somatic cells maintained their constant speed (Fig. 6A). The mean speed of exogenous PGCs increased significantly ($P < 0.05$) after they reached the anterior one third of the embryo, while that of somatic cells showed no significant change (Fig. 6B). Meanwhile, unlike exogenous PGCs, when PKH26-labeled DF-1 cells were transplanted, they maintained a constant speed until they reached the anterior one third of the embryo, as somatic cells did (Supplementary Fig. S2). Also, when we observed the morphological changes in the exogenous PGCs during their fast migration (Fig. 6), most of the cells exhibited successive contraction and expansion, that indicating active migration (Fig. 7).

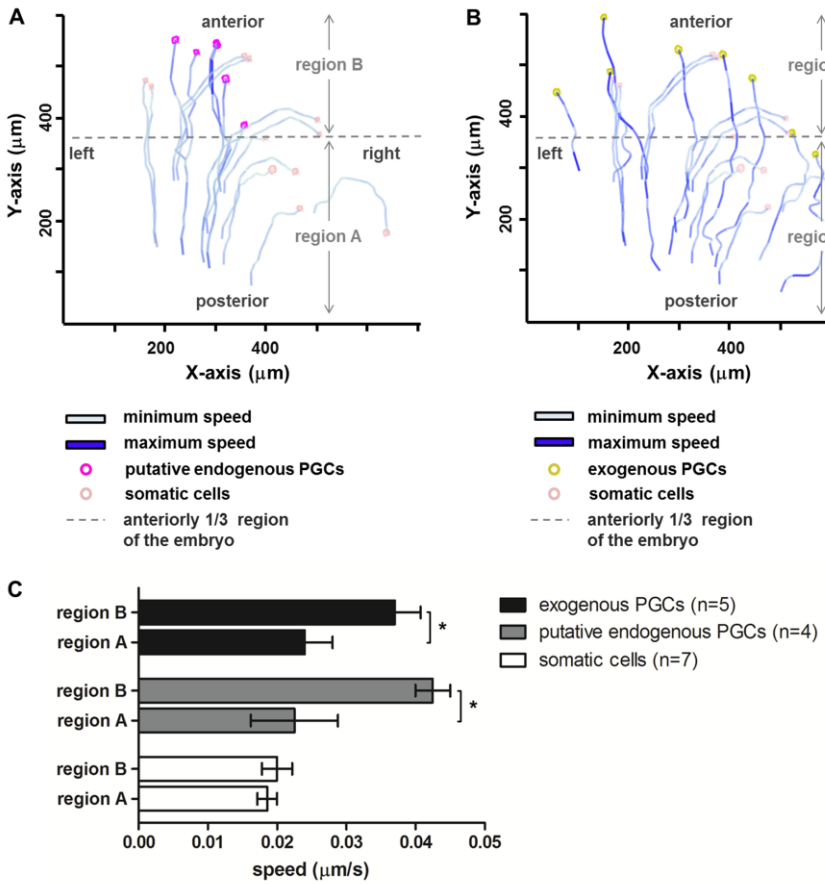

Figure 6. Space-specific increase in migratory speed in PGCs. Migratory tracks of PGCs compared to neighboring somatic cells were investigated between EGK stage X and HH stage 2 according to spatial and temporal criteria. **(A)** Line plots showing the migratory tracks of exogenous PGCs compared to neighboring somatic cells, indicating that PGCs increased their speed when they reached the anterior one third of the embryo. **(B)** The mean speed of exogenous PGCs was significantly higher in region B compared to region A, while the mean speed of somatic cells showed no significant change. The bars in **(B)** represent the SEM of five exogenous PGCs and seven somatic cells. **P* < 0.05, region A vs. B.

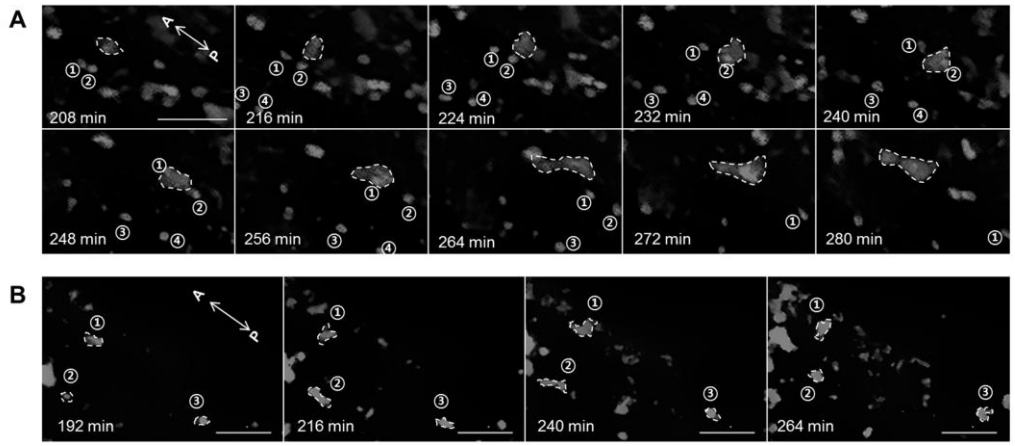

Figure 7. Amoeboid movement of chicken PGCs toward the germinal crescent. Morphological changes of PKH26-labeled exogenous PGCs in GFP-TG embryos were monitored between EGK stage X and HH stage 2. Magnified and sequential images of exogenous PGCs showing dynamic morphological changes and amoeboid movement toward the anterior region (dotted lines and circled number). For all images, the anteroposterior axis is from the upper left side to the lower right side. Scale bars = 100 μm.

Discussion

The migration pathway of chicken PGCs before they settle in the embryonic gonads has been determined by studying the relative positions of PGCs during embryonic development. PGCs located in the central region of the area pellucida during cleavage stages populate the germinal crescent, the anterior arc-like border region between the area pellucida and area opaca, at HH stage 4 (Fig. 8A), and migrate toward the genital ridges via the circulation (Fujimoto, 1976, Swift, 1914). However, details of the underlying mechanisms at each time point are largely unknown. In this study, we demonstrated the passive and active migration of PGCs, and the importance of PGCs' initial location in determining cell migration toward the germinal crescent.

First, we confirmed the previous idea that PGCs migrate passively by morphogenetic movement of the hypoblast (Ginsburg & Eyal-Giladi 1986). When transplanted into the subgerminal cavity in EGK stage X embryos, PGCs and somatic (DF-1) cells were located in the anterior border region at HH stage 4. These results indicate that when the cells land on the hypoblast, they are passively carried by anterior movement of the hypoblast, as PGCs are if they are translocated from the epiblast to the hypoblast (Swift 1914; Eyal-Giladi *et al.* 1981; Ginsburg & Eyal-Giladi 1986). Next, to further investigate the passive force toward the anterior region, PKH26-labeled exogenous cells were transplanted into the PMZ in EGK stage X embryos.

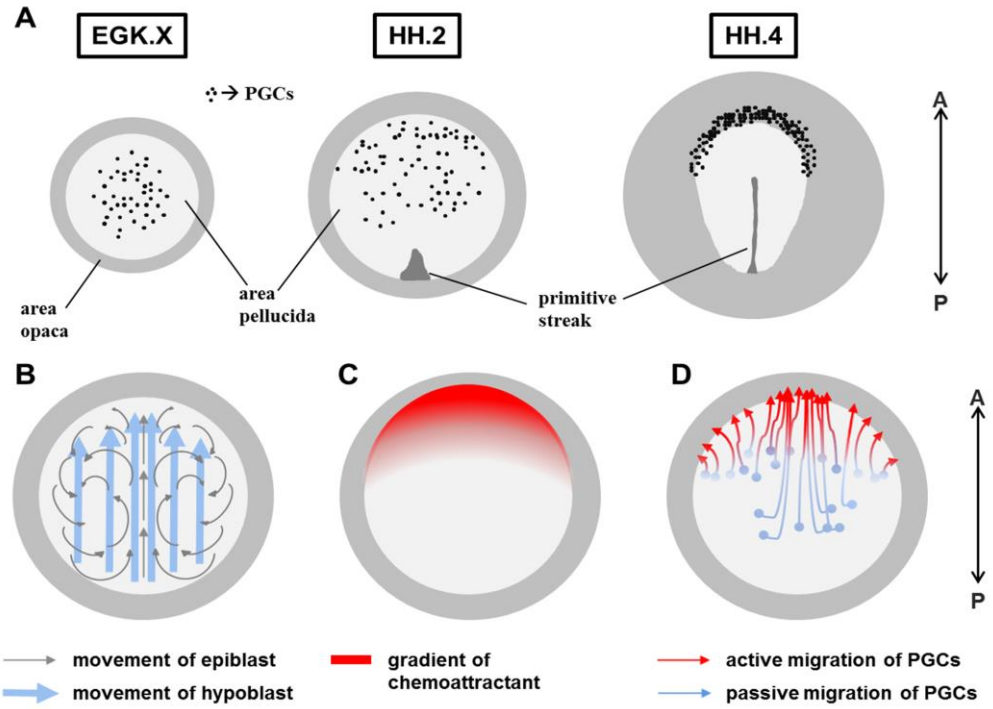

Figure 8. Proposed model for migration of chicken PGCs toward the germinal crescent. (A) Location of PGCs between EGK stage X and HH stage 4. At EGK stage X, PGCs are located in the central region of the area pellucida. By HH stage 4, most of the PGCs have reached the germinal crescent region. **(B)** Global movement of embryonic cells, including epiblast and hypoblast cells, between EGK stage X and HH stage 2. **(C)** Active migration zone between EGK stage X and HH stage 2, covering the anterior part of the embryo. **(D)** Migration pathway of PGCs toward the germinal crescent. Once PGCs reach the anterior region passively by embryonic flow (blue lines), they start actively migrating toward the germinal crescent region (red lines).

Notably, PGCs and DF-1 cells did not reach the anterior region when the embryos were incubated until HH stage 10, which indicates that for normal migration towards anterior region, the initial location of PGCs should be the central region of the embryo. In our study, PGCs transplanted into the PMZ did not migrate into the embryonic gonads. Because PGCs should be positioned in the anterior region before entry into the embryonic circulation to use the anterior vitelline veins (De Melo Bernardo *et al.* 2012), it seems reasonable that the site of PGC formation is the anterior region, not the central region as was thought. However, the anteroposterior (AP) axis does not develop at the time of PGC emergence in chicken. The c*VG1* gene, an initiator of axis formation, is known to be expressed from EGK stage X (Seleiro *et al.* 1996), and the first morphological indication of the AP axis formation of the area pellucida, is in EGK stage VII (Eyal-Giladi & Kochav 1976), although this is not well understood at the molecular level. However, chicken PGCs emerge at the initial cleavage stages (Tsunekawa *et al.* 2000).

We confirmed that PGCs migrate passively toward the anterior marginal region of the embryo during hypoblast formation and demonstrated the importance of the initial location of PGCs. In a previous study, however, PGCs near the germinal crescent around HH stage 4 showed an amoeboid morphology, indicating active cell migration (Ginsburg & Eyal-Giladi 1986). Therefore, to confirm whether migration was only passive, we magnified the germinal crescent region with immunostaining of endogenous PGCs. Only exogenous PGCs were located in the germinal crescent, where endogenous PGCs are positive for cDAZL. DF-1 cells were clearly located away from the germinal crescent. Also, many endogenous PGCs showed an amoeboid morphology at HH stage 4. These results indicate that although both PGCs and DF-1 cells can migrate passively toward the anterior region, only PGCs can gather in the germinal crescent by active migration. When the embryos were further incubated to HH stage 10 after cell transplantation, only exogenous PGCs were located in the anterior region of the area pellucida, where endogenous PGCs are positive for cDAZL. PGCs located in this area are ready to

enter the blood circulation through veins (Nakamura *et al.* 2007). However, most of the DF-1 cells were not located in this region. These results suggest that PGCs actively migrate toward the germinal crescent at HH stage 4 and the anterior region of the area pellucida at HH stage 10. To further clarify this point, molecular mechanisms governed by signaling should be investigated in further studies.

Based on our results, both passive and active forces are crucial for PGC migration. We further examined spatial and temporal aspects of passive and active migration through cell-tracking experiments with live imaging using GFP-TG. Centrally located exogenous PGCs at EGK stage X showed rapid movement toward the anterior border region, including both sides of the anterolateral regions, and most of them had already arrived at HH stage 2. When we checked the relative positions of endogenous PGCs during embryonic development by whole-mount *in situ* hybridization, many of them were still located in the central region of the embryo at HH stage 2 (others were in the germinal crescent). This discrepancy between exogenous and endogenous PGCs can be explained by the difference in their initial positions (Ginsburg & Eyal-Giladi 1986). Unlike the exogenous PGCs, which are located in the hypoblast under the epiblast after injection into the subgerminal cavity, endogenous PGCs migrate gradually from the epiblast towards the hypoblast until HH stage 4 (Ginsburg & Eyal-Giladi 1986). During their movement, PGCs exhibited morphological changes, with successive contraction and expansion, indicating active migration (Ridley *et al.* 2003). Endogenous somatic cells expressing GFP maintained normal epiblast movement (Hatada & Stern 1994). Also, the migration speed of the exogenous PGCs increased markedly after 4 h of incubation when the PGCs reached the anterior one third of the embryo. In contrast, somatic cells and DF-1 cells maintained a constant speed throughout the observation period. The dynamic increase in migration speed might be due to a gradient of chemoattractant spreading from the germinal crescent to the anterior one third of the embryo (Bianco *et al.* 2007; Mizoguchi *et al.* 2008) .

In this report, we propose a model for PGC migration toward the

germinal crescent. After specification, PGCs are located in the central epiblast or hypoblast until EGK stage X. Then, they migrate toward the anterior region passively by morphogenetic radial movement of epiblast or linear movement of the hypoblast in the posteroanterior direction (Fig. 8B). When the PGCs reach the anterior one third of the embryo, they migrate actively toward the germinal crescent with increased speed possibly in response to an unknown chemoattractant (Fig. 8C). We conclude that chicken PGCs use sequential passive and active forces to migrate toward the germinal crescent (Fig. 8D).

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CHAPTER 6

CONCLUSION

An embryo manipulation surrogate egg shell system is adapted in many different experimental fields. In 1988, Perry has contributed fertilized ovum culture system to produce chicks by ex ovo incubation. After this work, many scientists have published different studies describing embryo manipulation technology. However, these specified methods needs to be more simplified for improvement of viability.

The ICSI, which is an important technique in animal biotechnology for animal cloning and conservation of genetic resources, has been challenging in avian species. In this study, we investigated the ability of cryopreserved/thawed quail sperm to activate fertilization and embryo development. Female quail were sacrificed 70–120 min after previous oviposition to collect unfertilized oocytes from the oviduct.

Fresh or cryopreserved/thawed sperm were injected into the cytoplasm of unfertilized oocytes, and the manipulated oocytes were incubated in quail surrogate eggshells. The fresh sperm injection with inositol IP_3 showed significantly increased embryo development ratio when compared to fresh sperm only (90% vs 13%). Although more than 80% of the embryos stopped cell division and development before HH stage 3, approximately 15% of the embryos with the fresh sperm injection developed to over HH stage 4 and one embryo survived up to HH stage 39 (11 days of incubation). In the case of the cryopreserved/thawed sperm, embryo development ratio was 30% after ICSI and significantly increased to 74% when co-injected with IP_3. In conclusion, cryopreserved sperm combined with ICSI and following surrogate eggshell culture could develop quail embryos.

In most animals, PGCs originate from an extragonadal region and migrate across the embryo to the gonads where they differentiate and function. During their migration, PGCs move passively by morphogenetic movement of the embryo or move actively through signaling molecules.

To uncover the underlying mechanism of first-phase PGC migration toward the germinal crescent in chickens, we investigated the spatial and temporal action of PGCs during primitive streak formation. Exogenously transplanted PGCs migrated toward the anterior region of the embryo and the embryonic gonads when they were transplanted into the subgerminal cavity in EGK stage X embryos. But the exogenously transplanted PGCs into the posterior marginal zone was not migrated toward the anterior region of the embryo. These results indicate that the initial location of PGCs should be the central region for passive

migration toward the anterior region.

Notably, although PGCs and DF-1 cells migrated passively toward the anterior region, only PGCs migrated to the germinal crescent, where endogenous PGCs mainly reside, by active movement. In a live-imaging experiment with GFP-expressing transgenic embryos, exogenous PGCs demonstrated markedly faster migration when they reached the anterior one third of the embryo, while somatic cells showed epiblast movement with constant speed. Also, migrating PGCs exhibited successive contraction and expansion indicating their active migration. Our results suggest that chicken PGCs use sequential passive and active forces to migrate toward the germinal crescent.

As a result, we found that avian PGCs undergo passive migration toward the anterior region compared with the migration of somatic cells. Once PGCs reached germinal crescent in the anterior region, it starts rapid active migration then the constant migration of somatic cells.

In this study, we have simplified surrogate egg shell system and increased the hatchability in quail. We have produced high percentage of hatchability in quail egg shell surrogate system with both thick albumin capsulated and non-capsulated egg from freshly laid egg in 78% and 60%. Also, we simplified Q1 and Q2 system by adopting Ono's surrogate culture system and operated to cut off omphalo-mesenteric vein, while in hatching stage for increasing of hatchability. Furthermore, we have succeeded in higher percentage of hatchability in single cell stage of quail eggs collected from infundibulum and upper part of the magnum by surrogate egg shell incubation system.

In this study, we used Japanese quail as an experimental animal for simplifiing surrogate egg shell incubation when compared to previous complex manipulation systems in avian species. However, this unique technology requires complicated manipulation skill. Therefore, many scientific research groups were involved for their own distinctive experimental methods.

The aim of next study was to optimize the condition of cryopreservation and the concentration of cryoprotectant glycerol. We tested several thawing conditions and cryoprotectants. As a result, 8% glycerol mixed with 3% DMA in prefreezing diluents was a reasonable cryoprotectant that yielded 48% of motility ratio.

The prefreezing diluents containing 11% glycerol without DMA showed more

increased sperm motility. The mixture containing 6% glycerol and 5% DMA showed slightly less motility (43%) when compared to the mixture containing 8% glycerol. It would be easier to remove glycerol from the thawed semen in 6% glycerol mixture than 8% glycerol mixture. Thus, we used the prefreezing diluents containing 6% glycerol and 5% DMA for AI, and to fertilize the eggs.

To investigate the effects of glycerol removal by different incubation time (0, 10 and 30 min), the sperm surface was observed under scanning electron microscopy. Glycerol was not completely removed in the frozen-thawed sperm and sperm of 10 minute incubation.

In contrast, glycerol on sperm surface was not detected when it was incubated in thawing diluents for 30 min. These results showed that small amount of glycerol could simplify the removal process of glycerol. However, additional optimization and comparison among cryoprotectants are needed to be investigated.

In overall conclusion, in first part of this study, we efficiently developed surrogate egg shell system for the incubation and development of Japanese quail. Our surrogate egg shell system is much simple and showed higher percentage of quail viability and development when compared to slightly complex systems reported by other researchers. In second part of this study, we investigated ICSI with fresh, cryopreserved/thawed sperm for efficient fertilization of quail oocytes. The fresh or cryopreserved/thawed sperm coinjected with IP_3 gave significantly improved fertility when compared to injection of sperm without IP_3. At the end, we investigated initial migratory-phases of chicken PGCs which moving from the center of blastoderm to the germinal crescent area. We found that, initial migration of PGCs use passive and active forces of migration for moving from the central region to the anterior region where germinal crescent located. Also, we found that initial location of PGCs in the center of blastoderm is very important for undergoing passive and active migration. In third part of this study, we strongly believe that our surrogate egg shell system and ICSI techniques are much helpful for the conservation of avian species and improvement of avian genetic resources.

CHAPTER 7

SUMMERY IN KOREAN

조류는 체외에서 배아를 배양할 수 있는 가능성 때문에 많은 연구에 사용 되고 있다. 예를 들어 배아 발달 과정 실험, 형질전환 동물생산, 혈관형성 실험 등 수많은 학문에 이용되고 있다. 하지만 1988년 Perry에 의해 완성된 배양 기법이 많은 연구자에 의해 고도화 체계화 되었음에도 불구하고 많은 한계점을 안고 있다.

본 연구에서 사용된 실험은 대리난각 배양의 단순화와 체계화를 위한 실험적 검정을 위해 실시하였다. 실험의 진행은 산란된 알을 대리난간으로 옮겨 배양하였으며 체계화된 시스템 1-3 방법을 이용 하였다. 그러나 부화 시 죽게 되는 개체가 많이 발생하였다. 따라서 먼저 배양기의 난백의 점도에 따라 사용 목적을 달리 하여 사용하였다. 다음은 대리난각의 크기로 같은 크기의 난각을 사용 할 경우 부화가 되지 않았다. 본 실험에 사용 된 대리 난각은 14g으로 메추리가 생산 하는 알의 무게 중 상위 4% 내의 것을 사용하였다. 그리고 같이 진행된 초기 발생된 수정란의 대리난각 배양법의 완성을 위해 산란 시간이 조사된 메추리의 산란 시간에 맞추어 갓 수정된 난을 채취하고 이를 이용하여 대리난각 배양을 실시하였고 기술 수정된 대리난각 배양법에 의해 약 70%의 부화률을 보여 대리난각 배양의 상용화에 이용할 수 있는 가능성을 보였다.

유용유전자원의 보존은 동물 생명 공학에서 중요하지만 더디게 진행 되고 있는 학문 중 하나이다. 유용 유전자의 보존과 부활을 위한 연구는 많은 연구자들이 시간과 노력을 들여 진행하고 있다. 본 실험의 목표는 조류의 정자를 미세주입법을 이용하여 배아를 생산하는데 있다.

본 실험에서는 배란 후 70-120분된 메추리의 수란관에서 무정란을 수집하고 정자 미세 주입법을 이용하여 배아가 발달함을 확인하였다. 배아의 발달은 아주 낮은 수준에서 관찰 되었고 (13%), IP_3를 정자와 같이

주입함으로 수정률을 높일 수 있었다 (90%). 또한 동결해동 정자의 경우 약 74%의 수정률을 보여 정자미세주입법의 사용 가능성을 보여 주고 있다.

80년 이상의 연구가 진행 되고 있는 조류의 정자 동결 보존은 아직은 상업화 하기에 많은 문제점을 안고 있다. 이러한 문제점을 해결 하기 위해 다양한 동결보호제제가 사용 되고 있다. 예를 들어 DMA (Dimethylacetamide), DMSO (Dimethyl sulfoxide), DMF (Dimethylformamide), EG(Ethylene glycol) 가 사용되고 있으며 가장 널리 사용되고 있는 동결보호제는 glycerol이다. 그러나 glycerol의 경우 수정률과 발생률에 부정적 영향을 끼쳐 사용에 많은 어려움을 가지고 있다. 따라서 현재 진행되고 있는 동결 정자의 미세 주입법과 이를 배양 할 수 있는 기술의 조합은 조류의 종 보존과 재생산이라는 목표에 가까워질 수 있는 하나의 기술로써의 가능성을 보여 주고 있다.

본 실험에서 사용된 메추리의 경우 짧은 세대 기간과 작은 몸집으로 실험 동물로 사용에 적합성을 보여 주고 거의 매일 난을 생산 함으로 실험의 용의성을 가지고 있다. 메추리는 케이지 사육을 하였으며 산란 시간을 매일 측정하여 다음 산란 시간을 추정 하였다. 측정된 산란 시간에 맞추어 도축된 메추리의 몸에서 산란된 무정란에 정자와 IP3를 같이 주입함으로써 수정을 유도하였으며, 수정란을 대리난각에 옮겨 배양하였다. 결과적으로 최대 11일까지 배아 발달이 이루어졌으며 이는 조류 정자자미세주입법이 조류의 종 보존과 복원에 사용 가능함을 보여주는 결과로 판단할 수 있다. 또한 크게 차이 나지 않는 동결 정자의 수정률은 보존 후 복원이 가능 함을 보여 줄 수 있는 실험 결과를 보여 주었다.

조류 정액의 동결보존은 많은 기술적인 어려움 및 조류 난 산란 기관의 복잡성 때문에 80년 이상의 역사에도 불구 하고 상업화에는 많은 문제점을 가지고 있다. 본 실험에서 사용된 항 동해제는 DMA와 glycerol

로 현재 가장 널리 사용 되고 있는 항동해제이다. 본 실험에서는 항동해제를 섞어서 사용함으로 glycerol의 함량을 낮추고 항동해제로써의 기능을 향상 시키는데 있다. 최고의 혼합은 8% glycerol 3% DMA로 약 48%의 운동성을 보여 주었다 그러나 glycerol이 수정과 발생에 부정적 영향을 끼치는 경우가 많이 보고 되어 있고 실제 실험에서도 수정이 거의 되지 않았다. 따라서 최상의 조합은 6% glycerol과 DMA 5%를 이용한 것으로 약 43%의 운동성을 보여 주고 있다. 이를 이용한 실험을 통해 수정이 가능함을 확인하였다. 다음은 glycerol 제거를 위한 실험으로 glycerol 처리 후 전자현미경 사진을 통해 제거되는 과정을 관찰하였다. 실험 결과, glycerol 제거를 위한 시간은 30분 이상의 시간을 8배 희석된 배양액이 필요함을 관찰할 수 있었다.

조류의 원시 생식세포는 유전물질의 전달과정에 중요한 역할을 하고 있는 세포 중 하나이다. 따라서 많은 연구자들이 원시생식세포의 발생과 이동 기작을 연구 하기 위해 많은 노력을 기울여 왔다. 본 연구는 원시생식세포의 이동을 다각도의 실험을 통해 증명하였다.

조류의 원시생식세포 (Primordial germ cells, PGCs)는 낭배외피(epiblast)로부터 발달하여 생식선으로 이동하게 된다. 원시생식세포의 이동은 수동적 이동과 능동적 이동으로 이루어지며 포유류의 이동과는 차이를 보인다. 배반엽 단계의 원시생식세포의 이동은 낭배외피에서 유래하여 원시선이 형성되는 시기에 내배판으로 이동을 시작한다. 이 단계에서는 약 4-20개 정도의 원시생식세포를 확인 할 수 있다. 원시생식세포가 낭배외피로부터 분리되어 내배판으로 이동을 완료하는 시기는 stage8 에서 이루어진다.

원시생식세포 이동능의 기본 메커니즘을 밝히기 위해서 stags X 단계의 배아의 서브져미날 케비티에 왜래 형질전환 원시생식세포와 염색된

DF-1 세포를 이식하여 관찰 하였다. 초기 원시생식세포의 이동은 수동적으로 세포의 증식 방향과 일치하여 이동하다 져미날 크레센터의 위치 즉 배아의 3분의 1 지점을 통과하면서 능동적 이동을 보여 주게 된다. 이는 각기 다른 염색이 된 외래 형질전환 원시생식세포와 염색된 DF-1 세포의 이동 속도와 이동 경로를 광학현미경, 형광현미경 및 시간차 비디오 촬영을 통해 확인하였다.

이와 같은 실험을 증명하기 위해 먼저 *cDAZL* 염색을 이용한 홀마운트 인시츄 하이브리다이제이션을 실시하여 원시생식세포의 이동 경로를 확인하였다. 결과로 stage XII 에서는 원시생식세포의 위치가 중앙에 있음을 확인하였고 stage 2와 stage3에서 엔테리어 지역에 나타나기 시작해서 stage 5 에서 져미날 크레센터에 이동함을 확인하였다. 다음으로 PKH26으로 염색된 DF-1 세포와 외래 원시생식세포를 stags X 단계의 배아의 서브져미날 케비티에 주입시켜 이동 경로를 파악하였다. 두 세포 모두 엔테리얼 마지날 지역으로 이동함을 확인하였고 따라서 수동적 이동을 증명할 수 있었다. 같이 진행된 주입실험에서 포스테리얼 지역에 주입된 세포의 경우 엔테리얼 지역으로 이동하지 않음을 확인 하였다. 다음은 주입된 두 세포를 stage 4까지 배양하였을 때를 관찰하였고 *cDAZL* 염색을 이용하여 내부 원시생식세포의 위치와 외래 원시생식세포 그리고 DF-1 세포의 위치를 확인한 결과 내부 원시생식세포와 외래 원시생식세포는 져미날 크레센터에 위치함을 확인하였고 DF-1의 경우 위치하지 않음을 확인함으로써 능동적 이동을 증명하였다. 결과적으로 조류의 원시생식세포는 초기 단계에서 수동적인 움직임을 보이다 포스테리알 지역에 가까워짐에 따라 능동적으로 움직임을 알 수 있었다.

본 연구는 원시생식세포의 이동 경로를 추적하여 배 발달시 일어나는 일련의 과정 중 생식세포가 이동 하는 과정 중에 필요한 생체내 변화를 알아 봄으로써 발생학적 의미를 부여 할 수 기초자료로 사용될 수 있음

을 보여 주었으며 정자미세주입법을 이용한 조류 배아의 생산이 종의 보존과 생산에 도움이 될 수 있음을 보여 주고 있다. 일련의 과정을 통한 배아의 생산과 이를 위한 기초 초석인 정자의 동결 보존이 같이 맞물려 하나의 결과를 이루기 위한 실험이 되었음을 보여 주고 있다. 각 실험을 통해 조류의 수정과 발생 그리고 탄생까지의 일련의 과정을 이해하고 실험을 통해 검정함으로써 조류 발생의 전반적인 이해에 자료로 사용 가능할 것으로 판단된다. 또한 종의 발생에 대한 실험 보존이 필요한 유전 자원의 심도있는 연구와 보존 가능성의 제고에 많은 도움이 될 것으로 보인다.